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PARTIAL PURIFICATION AND PROPERTIES OF A "CORTICOTROPIN INFLUENCING FACTOR" (CIF) FROM HUMAN SPINAL FLUID: AN ASSAY METHOD FOR CIF IN THE TRAINED DOG.[1]

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ABSTRACT

By use of trained dogs with externalized carotid arteries an assay for corticotropin influencing factors has been developed. Injection of cerebrospinal fluid from man or from dog into the carotid artery of such dogs produced a detectable elevation of plasma 17-OHCS within 15 minutes, similar to that observed when 1 μg of ACTH per lb. of body weight was given. A satisfactory dose response curve was obtained when the increased level of 17-OHCS was measured as a function of the amount of spinal fluid (or purified factor) injected into the animal. The factor in spinal fluid can be purified approximately 25-fold by zone electrophoresis on a starch column or on paper. This factor appears to be protein or polypeptide in nature. Present information suggests that the factor may act to stimulate ACTH secretion through the parts of brain irrigated by the carotid artery.

A HYPOTHALAMIC neurohumor or neurohumors may act to release corticotropin (ACTH) (1). Saffran and Schally (2) found a corticotropin releasing factor (CRF) in neurohypophysial tissue of the rat, and Guillemin *et al.* (3) have isolated a substance from hypothalamus or the posterior part of the hypophysis which sets off ACTH discharge *in vitro*. The work of Guillemin *et al.* (3) indicates that this factor might be found in various parts of the brain. Fraction *D* from hypothalamic tissue has recently been shown to promote ACTH release in morphine-nembutal treated rats (4). Newman *et al.* (5) obtained a reduced secretion of cortisol in animals with destruction of the ventral diencephalon, and Royce and Sayers (6) have reported corticotropin releasing activity of a pepsin-labile factor

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in the hypothalamus. In contrast to pitressin (7), the hypothalamic material is not an ACTH-releasing factor in decapitated animals (8).

From the foregoing it appears that substances are elaborated by the hypothalamus and possibly by other parts of the brain which can promote the secretion of ACTH (9). This being the case, such substances might enter the cerebrospinal fluid either through diffusion or active secretion. The present study reports the partial purification and some properties of an active substance obtained from human cerebrospinal fluid. We also wish to report the development of an assay method which depends on the use of trained dogs. Changes in ACTH secretion are measured by estimating 17-hydroxycorticosteroids (17–OHCS) in plasma from animals which are injected with the test substances via the exteriorized carotid artery.

MATERIALS AND METHODS

Mongrel dogs of both sexes weighing 50–80 lbs. were used. The animals were kept in large, individual cages and fed Purina Dog Chow and water once a day. After deworming and treatment with distemper vaccine, the dogs were given a standard dose of 1.2 I.U. ACTH/kg. body weight intravenously and plasma levels of 17-hydroxycorticosteroids (17-OHCS) were measured 2,4, and 6 hours after the injection. Animals exhibiting a normal ACTH response (10) were selected for further use. In these animals the left carotid artery was externalized as described by van Lersum (11) and by Verney (12). Three to four weeks postoperatively the functional ACTH test was repeated and animals responding normally were selected for subsequent training. This consisted of training the animals twice a week to lie down on the laboratory floor on command and sit up with the neck erect when ordered to do so. When this was achieved, three samples of 30 ml. blood each were removed from the leg vein at 10-minute intervals. Plasma 17-OHCS were determined in these samples. During the early phase of training, an increase in the concentration of these steroids were found over the 30-minute period. After 3 to 4 months training with withdrawal of blood the animals apparently accommodated to this "stress" condition since very little fluctuation in plasma 17-OHCS was seen. The carotid "loop" was then punctured and 5 ml. of sterile 0.85% sodium chloride at 37.5° C were injected over a 15-second period. Again, this treatment elicited a response in plasma 17-OHCS. Training was then continued until this response disappeared.

After training the dogs every week for 8 to 10 months, in the fashion outlined, the "loop" dogs exhibited only a very slight response in plasma 17-OHCS when subjected to the following procedure.

The animals were brought without a leash from their quarters in the basement to the laboratory on the second floor. A blood sample of 30 ml. was taken for the determination of plasma 17-OHCS and 5 ml. of isotonic saline at 37.5° C were injected into the carotid loop over 15 seconds. The dog was ordered to lie down and another 30 ml. of blood was removed 15 minutes after the intracarotid injection. If the concentration of plasma 17-OHCS in the last sample was not more than 30–50% higher than in the control sample, the animal was considered suitable for experimental use. Only 56% of the operated dogs survived to serve as suitable test animals.

All the experiments reported were done between 1:00 P.M. and 5:00 P.M. The levels of 17-OHCS in dog plasma are lower at this time of day than during the early morning hours and also undergo less fluctuation (13). Plasma levels of 17-OHCS were determined as described by Eik-Nes (14). Several experiments were also performed in normal guinea pigs by measuring plasma 17-OHCS (15) and in hypophysectomized rats by the adrenal ascorbic acid depletion method (16).

Spinal fluid was collected from the 3rd ventricle and the preoptic subarachnoid region of normal, anesthetized dogs. These fluids were kept at 4° C for no longer than 12 hours before being injected into the test animals. Human spinal fluid was obtained by spinal tapping from patients shortly before surgical operation. This spinal fluid was pooled from numerous patients and kept in a deep freezer before being assayed or subjected to purification. Since it was observed that loss of activity occurred with spinal fluid as well as with partially purified preparations after repeated freezing and thawing, these processes were kept to a minimum and the various preparations were administered to test animals as soon as possible.

RESULTS

Assay Method.—Fifteen minutes after the intravenous injection of 1 μg ACTH/lb.[2] body weight into the leg vein, plasma levels of 17-OHCS had

TABLE 1. PLASMA LEVELS OF 17-HYDROXYCORTICOSTEROIDS (μG./100 ML) IN TRAINED "LOOP" DOGS*

No. of tests	Condition	17-OHCS (μg/100 ml.)	
		Before injection	15 minutes after injection
21	5 ml. .85% NaCl	1.8 ± .10	2.3 ± .21
7	1 μg ACTH/lb. in 5 ml. .85% NaCl (given by the leg vein)	1.5 ± .13	5.0 ± .47
8	1 μg ACTH/lb. in 5 ml. .85% NaCl	1.6 ± .12	4.9 ± .20
13	5 ml. dog spinal fluid obtained from the preoptic-subarachnoid region	1.9 ± .10	5.7 ± .30
8	5 ml. human spinal fluid (given by leg vein)	1.6 ± .10	2.0 ± .21
12	5 ml. human spinal fluid	1.9 ± .12	4.6 ± .30
6	2 μg partially purified spinal fluid protein/lb. in 5 ml. .85% NaCl (given by leg vein)	1.9 ± .24	2.2 ± .25
5	2 μg partially purified spinal fluid protein/lb in 5 ml. .85% NaCl	1.7 ± .20	4.9 ± .32
8	Homogenate of 1000 μg dog corpora mammilaria in 5 ml. .85% NaCl	1.9 ± .19	2.6 ± .26
6	1 μg human plasma γ-globulin, Fraction II_3/lb. in 5 ml. .85% NaCl	1.8 ± .26	2.0 ± .22
8	1 μg beef growth hormone/lb. in 5 ml. .85% NaCl	1.6 ± .09	2.3 ± .17
6	1 μg lactogenic hormone/lb. in 5 ml. .85% NaCl	1.9 ± .22	2.2 ± .30
7	1 μg ICSH/lb. in 5 ml. .85% NaCl	1.4 ± .36	1.7 ± .29

* All levels are mean levels with one standard error of the mean. If not otherwise indicated, the material was injected via the externalized carotid artery of the trained animal.

increased significantly in the dogs (Table 1). When this dose of ACTH was given by the carotid loop, the increase in plasma 17-OHCS was the same as when the hormone was given by the leg vein (Table 1). An increase of this order in the plasma 17-OHCS concentration was, therefore, assumed to be indicative of adrenal stimulation.

Five ml. of human cerebrospinal fluid obtained by spinal tap were warmed to 37.5° C and injected into the carotid loop in the standard way. An increase in plasma 17-OHCS similar to that obtained by injecting ACTH was seen in all animals tested (Table 1). When this amount of spinal fluid was given intravenously by the leg vein, no significant altera-

[2] 1 mg. of this material contained 110 I.U. of ACTH.

tion in the level of plasma 17-OHCS was detected (Table 1). The response when spinal fluid was given by the carotid artery indicated therefore that this material influenced the metabolism of ACTH centrally. Active material was found in dog spinal fluid obtained from the third ventricle, the lumbar subarachnoid space and the subarachnoid space in the preoptic region (Table 1).

Figure 1 shows the results of several assays in which the response of plasma 17-OHCS was measured as a function of the amount of protein from human spinal fluid which was administered via the carotid loop of

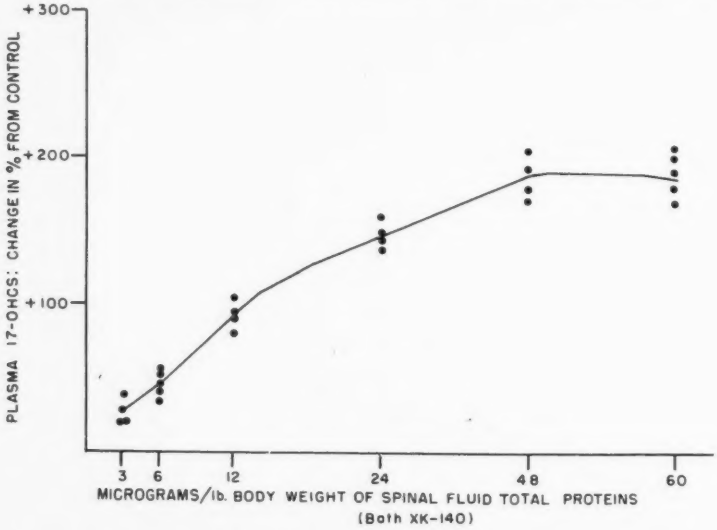

FIG. 1. Change in plasma 17-OHCS 15 minutes after the intracarotid administration of varying amounts of human spinal fluid protein.

the trained dog. It is evident that a graded response is obtained and that the maximal level, 200% above that in the uninjected dog, is in accord with previous studies in such animals (17).

Purification.—For further study of the active material, 200 ml. aliquots of pooled human spinal fluid were placed in a dialysis bag, and pervaporation was performed by blowing air on the outside of these bags with a standard floor type fan at room temperature. This treatment reduced the volume of fluid to 2–3 ml. which contained a total of 50–70 mg. protein (18). The fluid was centrifuged and the insoluble fraction was separated. The insoluble material, when dissolved in saline and injected in the test animals, had no effect on the levels of plasma 17-OHCS.

The supernatant fluid from the centrifugation was then dialyzed against a 0.05 M phosphate buffer at pH 8.0. After dialysis, the protein solution was applied to the vertical starch column described by Hill *et al.* (19).

Electrophoresis was performed for 24 hours at 30 milliamps. in 0.05 M phosphate buffer at pH 8.0. Elution of the separated fractions was effected with the same buffer and 1 ml. fraction were collected with the aid of a Technicon time-flow fraction collector. The protein concentration in each tube was determined by a tubidometric method (18). A typical pattern is shown in Figure 2.

Pools of the contents of several tubes were made as indicated in Figure 2. From each pool 200 μg of protein were dissolved in 5 ml. of 0.85% sodium chloride, warmed to 37.5° C and injected into the carotid artery of the

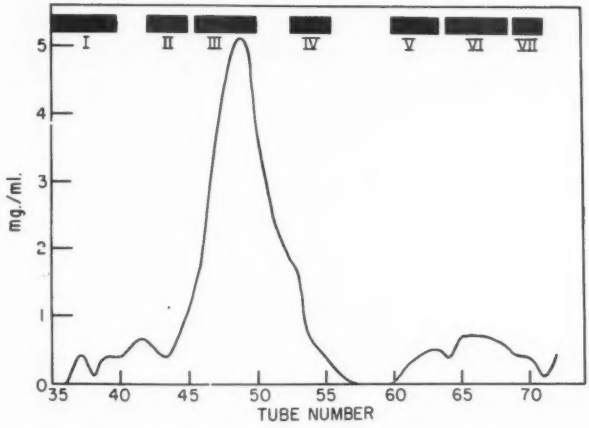

FIG. 2. Concentration of protein as a function of elution volume after electrophoresis on a starch column of concentrated human spinal fluid protein. The roman numerals indicate the pools which were tested for activity. Active material was found only in Fraction VI.

trained dogs. At this level activity could be found only in the pool of Tubes 64–68 (Fraction VI). When different doses of this material were tested, 2 μg/lb. gave a maximal response as indicated by the data in Figure 3. It is noteworthy that the form of the curve in Figure 3 is similar to that shown in Figure 1 for whole spinal fluid protein. Comparison of the data for the crude and partially purified material indicates a purification of about 24-fold. As indicated by the data in Figure 1, it required 12 μg/lb. to produce a 100% increase in steroids whereas it required only about 0.5 μg/lb. to obtain the same response with Fraction VI (Fig. 3).

To obtain larger amounts of the active material the Spinco CP Continuous Flow Paper Electrophoresis Apparatus was utilized. The spinal fluid, after concentration by pervaporation, was dialyzed against 0.02 M phosphate buffer at pH 8.0 for 24–48 hours before being applied to the curtain. Flow rates were adjusted by setting to 3.5 on the upper curtain and 9.5–10.0 or 1 drop per 10–15 seconds on the side wicks. Application of

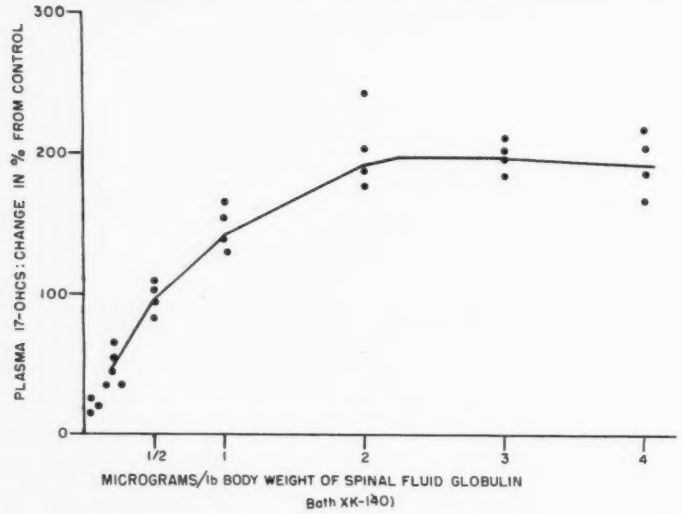

FIG. 3. Change in plasma 17-OHCS after the intracarotid administration of varying amounts of the active fraction of spinal fluid protein partially purified by starch column electrophoresis.

the sample was maintained at 1 to 1.5 ml/hour, and operation of the continuous flow apparatus was continued for 18 to 27 hours at a constant current of 60 milliamps. requiring approximately 400 volts. The same degree of purification and overall yield resulted from this treatment as from electrophoresis on the starch column. A representative pattern is shown in

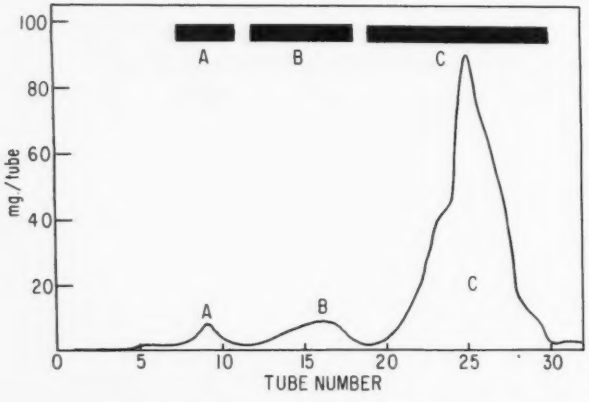

FIG. 4. Concentration of protein as a function of tube number after subjecting concentrated human spinal fluid protein to electrophoresis by the continuous flow method. All of the activity was found in Fraction A.

Figure 4 where concentration is given versus tube number. It has been found that more than 200 mg. of protein, representing almost one liter of spinal fluid, can be fractionated by the procedure.

Additional Tests.—Inasmuch as the active fraction obtained from the spinal fluid emerged in the region expected for γ-globulins, human plasma γ-globulin, (Fraction II-3) at a dose of 1 μg/lb. was tested and found to be ineffective (Table 1). Several anterior pituitary hormones were tested at the same dose level and also found to be inactive (Table 1). Finally, no increase in plasma 17-OHCS was found when 1000 μg of corpora mammilaria from the dog were homogenized in saline, filtered and the supernatant solution administered.

When 2 μg of purified spinal fluid globulins were given per lb. of animal by the leg vein, no significant response of plasma 17-OHCS was seen (Table 1). This indicates that the spinal fluid material produced its effect by an action in the central nervous region rather than in the adrenals.

A dose of 1.5 μg/gram of body weight of purified spinal fluid globulins was injected into guinea pigs weighing from 280–400 grams. As shown in Figure 5 the level of the plasma 17-OHCS was increased and reached a maximal value in 2 to 3 hours.

When one μg/gram of the partially purified spinal fluid material was given to rats hypophysectomized 23 hours earlier, no depletion of adrenal ascorbic acid could be found (Fig. 6), indicating again that the material did not act directly on the adrenal cortex.

The spinal fluid material was tested for vasopressin activity in the rat.

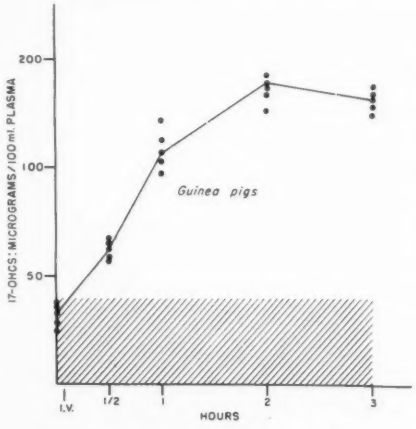

FIG. 5. Plasma levels of 17-OHCS in normal guinea pigs after the intravenous administration of 1.5 μg/gm. of partially purified spinal fluid protein. The shaded area indicates the control range.

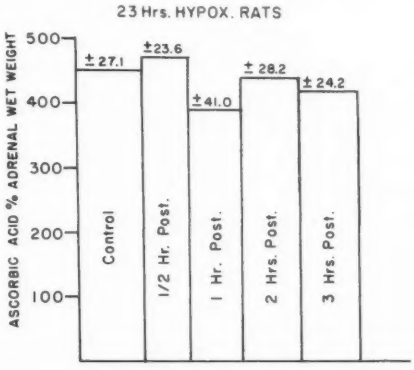

FIG. 6. Mean concentrations of adrenal ascorbic acid in hypophysectomized rats after the injection of partially purified human spinal fluid protein. Each column represents 4 animals. The numbers at the top of each bar represent the standard deviation of the mean.

When compared to U.S.P. XV posterior pituitary standard the spinal fluid preparation contained less than 0.008 pressor units/mg.[3]

Isoelectric Point.—Purified samples of pooled, active fractions from either starch column or curtain electrophoresis were subjected to paper strip electrophoresis with the aid of the Spinco model R electrophoresis apparatus. Figure 7 is a plot of distance moved versus pH. Normal human serum was run simultaneously as a control and the values for the albumin are plotted for comparison. The apparent isoelectric point was at pH 5.4. The control albumin showed an isoelectric point at pH 4.7, in good agreement with the known value for this protein (20).

The paper electrophoresis pattern obtained at pH 4.0 indicated some polydispersity. Several subsequent runs were performed at this pH and

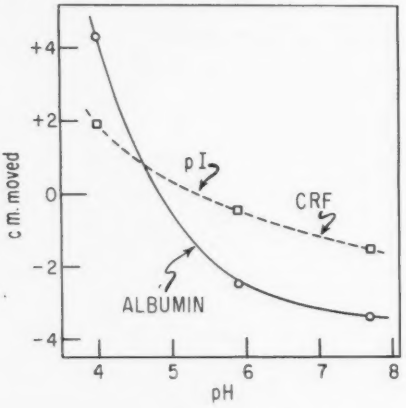

FIG. 7. Electrophoretic mobility as a function of pH for the active spinal fluid fraction and for a control of human serum albumin.

samples were eluted from the paper strips. Three possible fractions were obtained corresponding to a portion remaining at the origin, a low moving component and a faster moving component. These three fractions were then tested for activity in the assay animal. The most rapidly migrating fraction was found to be active whereas the other fractions were not. This suggests a procedure by which it may be possible to achieve further purification.

Sedimentation Studies.—Purified preparations obtained by starch column electrophoresis were equilibrated by dialysis against 0.1 M veronal buffer at pH 8.5 or against 0.1 M phosphate buffer at pH 7.0 and then subjected to high speed centrifugation at 59,780 RPM at room temperature in the Spinco ultracentrifuge. The sedimentation pattern indicated the presence of two major components, one having a sedimentation constant of approximately 2.2.–2.4 Svedberg units representing approximately 35% of the material and the heavier component of 4.8–5.5 Svedberg units making up

[3] We are indebted to C. E. Powell of the Eli Lilly Company, Indianapolis, for this assay.

the remaining 65% of the sedimenting material. These values would correspond to molecular weights of approximately 20,000 and 80,000 respectively, if it is assumed that the diffusion constants and the partial specific volumes are similar to those of other globular proteins. It is noteworthy that although the active material migrates in the γ-globulin range of electrophoretic mobilities the partia y purified preparation contains no γ-globulin as judged by the sedimentation behavior.

Stability.—As indicated in Table 2, the partially purified material from spinal fluid retains activity after a short exposure to acid or base. Heat and digestion by pepsin or trypsin destroy all activity indicating that the factor is probably a protein. Furthermore, a considerable loss of activity is observed after repeated freezing and thawing or after storage at 0–5° C for long periods.

TABLE 2. STABILITY OF ACTIVE MATERIAL FROM HUMAN SPINAL FLUID INFLUENCING THE METABOLISM OF ACTH. TWO μG/LB. WERE INJECTED BY THE CAROTID "LOOP" OF TRAINED DOGS AND EACH PREPARATION WAS TESTED IN 3 ANIMALS

Condition	Activity
1. Heated at 60° C for 15 min.	Full activity
2. Heated at 95° C for 10 min.	Complete loss of activity
3. Exposed to 1.0 M HCl for 1 hr. and then neutralized with 1.0 M NaOH	Ca 15% loss of activity
4. Exposed to 4.35 M acetic acid for 1 hr. and then neutralized with 4.35 M NaOH	Full activity
5. Exposed to 1% trypsin for 24 hrs. at 24° C	Complete loss of activity
6. Exposed to 1% pepsin for 24 hrs. at 24° C	Complete loss of activity
7. Kept frozen for 9 months	50% loss of activity

DISCUSSION

In the studies given above a procedure is described which permits an assay of ACTH secretion under as physiological conditions as possible. The assay gives a satisfactory dose-response curve as shown by the data of Figures 1 and 3 for crude and partially purified samples respectively. The use of trained animals circumvents many problems although, admittedly, the procedure is very time-consuming.

It has been demonstrated that spinal fluid contains a factor which augments secretion of ACTH, as judged by the adrenal response. That this factor is not ACTH itself is indicated by the finding that *the factor does not deplete adrenal ascorbic acid in hypophysectomized rats.* The factor is not vasopressin as judged by assays on the partially purified material. When a total dose of 200 μg of the partially purified material-containing *a total concentration ca 2 milliunits vasopressin* was given by the "loop" an ACTH like response was seen. It is not likely that this is due to the presence of vasopressin, inasmuch as we have observed that after the administration of L_8 vasopressin[4] by the "loop" in the trained dog, a dose of between 2–4

[4] Obtained from Sandoz Pharmaceuticals, Hanover, New Jersey.

milliunits per lb. or *a total dose of 100–400 milliunits* L_8 vasopressin is required to elicit an ACTH like effect.

There appear to be several alternative possiblities as to the action of the spinal fluid material: (A) that it acts as CRF (2); (B) that the factor may influence the central nervous system and stimulate the secretion of ACTH by the same mechanisms as other corticotrophin influencing factors (CIF); and (C) that the factor acts as an inhibitor of a pituitary proteinase thus inhibiting ACTH destruction.

We have not presented adequate data in support of the view that the factor is CRF, since no experiments have been done in animals with hypothalamic lesions. Whether a morphine-inhibited animal may substitute as a test preparation for an animal with distinct, localized lesions in the hypothalamus (6) is unknown. Furthermore, we have not performed *in vitro* studies with partially purified material utilizing the standard CRF technique (2).

It is most likely that the partially purified material from human spinal fluid promotes secretion of ACTH by the brain tissues irrigated by the carotid artery, thus being a CIF. Such factors are known, e.g., histamine, epinephrine, bacterial pyrogens, vasopressin and 5-hydroxytryptamine, the last being also able to stimulate the adrenal cortex directly (7, 21). However, if such factors are permitted to reach the target organs in the central nervous system directly, they may promote ACTH secretion without necessarily changing the general body metabolism. Thus, in the case of vasopressin it has been demonstrated recently that this substance will promote the secretion of 17-OHCS when administered into the third ventricle of the dog (22). Ten times the dose effective by this route of administration had no effect on 17-OHCS secretion when given by the leg vein. Our trained animal preparation permits an adequate evaluation of corticotropin influencing factors, since the animals are not "stressed" in contrast to animals subjected acutely to anesthesia and surgery, the material in question (CIF) can be delivered directly to the tissues irrigated by the carotid artery, and a dose difference between systemic stimulation and localized stimulation can be established. The mechanism of action, hormonal-or neurogenic-, of CIF is not known. The presence of such material in human spinal fluid may indicate that the material may be involved in the secretion of ACTH under physiological conditions. On the other hand, this material may also be an excretion product in normal brain metabolism without any physiological significance. It is, however, of considerable interest to note that Shibusawa *et al.* (23, 24) consistently find a thyrotrophic releasing factor in the spinal fluid of the dog. The concentration of this factor decreased markedly in the spinal fluid after lesions were produced in the anterior hypothalamus (24). Thus active material involved in another function of the anterior pituitary gland is present also in the spinal fluid.

There is, at present, no evidence which indicates whether or not the active factor has any influence on pituitary proteinase. The marked sensitivity of the active factor to proteolytic destruction by trypsin and pepsin suggests that it is probably sensitive to other proteinases as well. The fact that the active factor promotes a rapid adrenal response, detectable in 15 minutes and possibly in even a shorter time period, also suggests an effect on ACTH secretion rather than a sparing action.

The data indicate that the crude material in human spinal fluid effecting the central metabolism of ACTH in the dog probably is a protein or a polypeptide. This is suggested by the observations of inactivation by proteolytic enzymes and by its thermal lability. In addition, the active material is not dialysable and it can be sedimented in the ultracentrifuge. Also, the active material behaves as an ampholyte with an apparent isoelectric point at pH 5.4.

Acknowledgment

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THE EFECTS OF ANALOGUES OF THYROXINE ON THE GLYCOLYSIS AND OXYGEN UPTAKE OF ASCITES TUMOR CELLS[1]

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ABSTRACT

A number of analogues of thyroxine which stimulate aerobic glycolysis in Ehrlich ascites tumor cells have been studied. The most active compounds contained at least three iodine substituents and an acetic or propionic side chain (triiodothyroacetic acid, tetraiodothyroacetic acid and tetraiodothyropropionic acid). Activity was greatly reduced when iodine was replaced by bromine and was virtually abolished by the presence of amino or keto groups in the side chain. Stimulation of aerobic glycolysis by these compounds was observed only with intact cells and was associated with a decrease in oxygen consumption.

The related compound, 2:6 diiodophenol, was a more potent stimulant of aerobic glycolysis than the above analogues and caused no depression of oxygen uptake. Substitution in the 4 position of this compound reduced its activity.

The mechanism of these various effects is discussed.

IN A previous study it has been reported that triiodothyroacetic acid (Triac) in vitro increases aerobic glycolysis and depresses oxygen consumption in Ehrlich mouse carcinoma ascites tumor cells (1, 2).

The present work was undertaken with two objectives in mind. The first was to explore how structural modifications of the Triac molecule would alter these effects. A number of compounds were tested in which the halogen substituents and side chain structure were varied. Several iodinated phenols were also tested. The second objective was to study the mechanism by which the Triac effects were produced. For this purpose, intact and broken cell preparations were employed.

METHODS AND MATERIALS

Intact Ehrlich Ascites tumor cells were obtained as previously described (1). The measurements of glycolysis and oxygen consumption were carried out by conventional manometric procedures using the Warburg Apparatus.

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[3] Supported by an Institutional Grant to Vanderbilt University from the American Cancer Society.

[4] Scholar of the American Cancer Society during the course of this work.

Homogenates of the ascites tumor cells were prepared from a 50% suspension of cells in a buffer containing 0.132 M KCl, 0.0138 M NaCl, 0.0042 M K_2HPO_4, 0.00138 M $MgSO_4$, 0.018 M nicotinamide and 0.0012 M Na ethylenediaminetetraacetic acid (final pH 7.3), by disrupting with a Servall Omnimixer at full speed for 3 minutes at 0°. The homogenate was spun at 600×g for 10 minutes at 0° in order to remove nuclei, debris and unbroken cells before testing. The mitochondria were obtained from this supernatant fraction by centrifugation at 9,000×g for 20 minutes and were washed with buffer before the manometric measurements. The supernatant fraction from the 9,000×g centrifugation was free of mitochondria as shown by the absence of respiratory activity but contained microsomes and soluble enzymes.

Glucose was determined by the method of Nelson (3) and lactic acid according to Barker and Summerson (4).

Most of the compounds studied were generously supplied by Dr. R. Pitt-Rivers. Samples of Triac and thyroxine were kindly supplied by Smith, Kline and French Co. DPN and ATP were obtained from Pabst Co. and cytochrome c from Sigma Chemical Co.

EXPERIMENTAL RESULTS

Effects of Triac. The typical effects of Triac on aerobic glycolysis and respiration are shown in Figure 1. The compound at a concentration of 50 μg per ml (8×10^{-5} M) stimulated glycolysis to 200% of the control rate and inhibited the oxygen consumption by 50%. The effect on glycolysis was immediate, and the increase in CO_2 production remained linear with time. The depression of oxygen consumption was noted in the first ten minute reading and became more pronounced with time. With Triac and

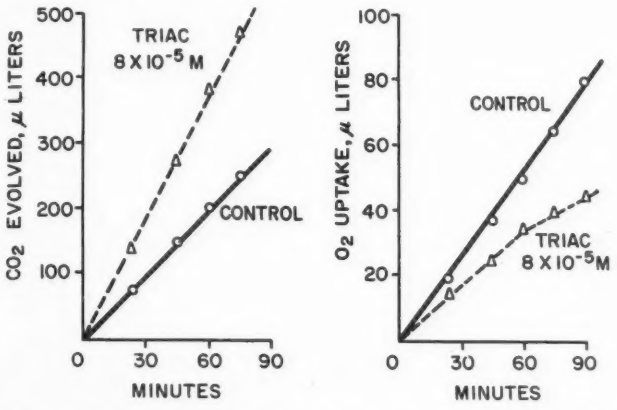

FIG. 1. *Effect of Triac on the aerobic glycolysis and oxygen consumption of Ehrlich ascites tumor cells.* Aerobic glycolysis was measured in the conventional Warburg apparatus in a Krebs-Ringer bicarbonate buffer, pH 7.4, gassed with 95% O_2−5%. Oxygen consumption was determined manometrically in a Krebs-Ringer phosphate buffer, pH 7.4, in air. 1 ml of a 10% suspension of cells was used in these experiments. 20 μ moles of glucose and 100 μg (0.16 μ moles) of Triac were placed in the side arm of the vessel. After ten minutes equilibration at 37° a zero time reading was taken, and the contents of the side arm were tipped into the cell suspension. Total volume was 2.0 ml.

the other analogues, stimulation of glycolysis was found to increase to a maximum at 8×10^{-5} to 1.6×10^{-4} M and then to decline as the concentration was raised further (1).

The measurements of glucose disappearance and lactic acid formation are compared with CO_2 production as shown in Table 1. The values for glucose disappearance and lactic acid formation are in the appropriate range to indicate that the major portion of the glucose is utilized through the glycolytic pathway. The lactic acid formation is somewhat lower than the CO_2 production due probably to the release of hydrogen ions from a variety of metabolic products. It should be noted that with Triac the percent stimulation of CO_2 production corresponds to the percent increase in lactic acid formation. The percent stimulation is low in this experiment

TABLE 1. THE CORRELATION BETWEEN INCREASED CO_2 PRODUCTION AND LACTIC ACID FORMATION

The experimental conditions for measuring CO_2 production were the same as described in Figure 1. The manometric measurements were made over a period of ninety minutes. The glucose (3) and lactic acid (4) determinations were carried out on suitable aliquots at the end of the manometric run.

Experimental conditions	Glucose uptake μ moles	CO_2 production μ moles	Lactic acid μ moles
Control	11.3	25.0	17.5
Triac (1×10^{-4} M)	18.6	42.8	32.0

as the time for manometric measurements was increased to give suitable values for the chemical determinations.

Effect of structural modification on Triac activity. The effects of structural modifications on the above activities are shown in Table 2.

1) *Halogen substitution.* The most active analogue was Triac which increased aerobic glycolysis to 195% of the control rate and depressed oxygen consumption to 60 percent. With only 2 iodine atoms in the molecule, the analog 3:5 diiodothyroacetic acid was virtually inactive towards aerobic glycolysis, but still had a considerable effect on oxygen consumption. When bromide was substituted for iodine (tribromothyroacetic acid), there was only a 45% stimulation of aerobic glycolysis. When one of these bromines was replaced by iodine there was no change in activity, but when two iodines and one bromine atom were present (3:5 diiodo-3′-bromothyroacetic acid) the activity toward glycolysis was increased.

2) *Side chain substitution.* The effect of side chain substitution was studied using a group of tetraiodo-substituted analogues. The activities of the tetraiodothyroacetic- and propionic-acids on aerobic glycolysis were the same as those of Triac. However, with a three carbon side chain the presence of an amino group (thyroxine), an N-acylated derivative (N-propionyl thyroxine) or a carbonyl group (tetraiodothyropyruvic acid) in the side

TABLE 2. THE EFFECT OF STRUCTURAL MODIFICATIONS OF TRIAC ON THE AEROBIC GLYCOLYSIS AND OXYGEN CONSUMPTION OF EHRLICH TUMOR CELLS

The experimental conditions were the same as in Figure 1. The concentrations of the various derivatives are given in the table. The calculations are based on an interval of 60 minutes.

Compound	Halogen content	Concentration*		Aerobic glycolysis % of control	Oxygen consumption % of control
		μg/ml.	Molarity		
Triiodothyroacetic Acid	3 I	50	8×10^{-5}	195	60
3:5-Diiodothyroacetic Acid	2 I	50	1×10^{-4}	118	70
Tribromothyroacetic Acid	3 Br	50	9.8×10^{-5}	145	60–50
3:5-Dibromo-3′-Iodothyroacetic Acid	1 I+2 Br	50	8.9×10^{-5}	145	60–50
3:5-Diiodo-3′-Bromothyroacetic Acid	2 I+Br	50	8.3×10^{-5}	160	70
	Side chain substitution				
Tetraiodothyropropionic Acid	$-CH_2CH_2COOH$	50	6.5×10^{-5}	190	60–50
Tetraiodothyroacetic Acid	$-CH_2COOH$	50	6.7×10^{-5}	191†	—
Thyroxine	$-CH_2CH(NH_2)COOH$	50	6.4×10^{-5}	100	80
Tetraiodothyropyruvic Acid	$-CH_2C(=O)COOH$	50	6.4×10^{-5}	120–130	—
N-Propionyl Thyroxine	$-CH_2CH(NH-COCH_2CH_3)COOH$	50	6.0×10^{-5}	129	60–50
	Iodinated phenols				
2:6-Diiodophenol		50	1.4×10^{-4}	210	100
		100	2.8×10^{-4}	238	65
3:5-Diiodo-4-hydroxyphenylacetic Acid		50	1.2×10^{-4}	130	70
Diiodotyrosine		50	1.1×10^{-4}	100	85
N-Propionyldiiodotyrosine		50	1.1×10^{-4}	100	100

* The exact concentrations of thyroxine, tetraiodothyropyruvic acid and N-propionyl thyroxine in solution during the course of an experiment were difficult to determine owing to the limited solubility of these compounds. This was especially true in the case of the pyruvic acid analogue.

† These data are from previously reported experiments (1) and were not corrected for oxygen consumption.

chain greatly reduced the activity. It can be seen that both thyroxine and the N-propionyl derivative inhibited oxygen consumption.

Experiments carried out with commercial samples of thyroxine of unknown purity often showed a stimulatory effect on aerobic glycolysis, probably due to contaminating material. Maley and Lardy have also obtained variable results using different samples of thyroxine (5). Perhaps impurities in thyroxine preparations may also account for the slight stimulatory effect on aerobic glycolysis observed by Barker and Lewis (6).

Higher concentrations (200 μg/ml.) of recrystallized thyroxine produced no stimulation of aerobic glycolysis but increased the inhibition of oxygen uptake to about 30 percent of the control rate.

3) *Iodinated phenols.* It was thought of interest to study the effects of different moieties of some of the iodothyronine analogues in order to determine if possible which part of the molecule was exerting the effect. Accordingly 2:6-diiodophenol, 3:5-diiodo-4-hydroxyphenylacetic acid and diiodotyrosine were tested. 2:6-diiodophenol was the most active compound in stimulating aerobic glycolysis but had no effect on respiration at a concentration of 50 μg/ml. This compound was the exception to the finding that increased glycolysis was related to a decrease in oxygen consumption. However, 100 μg/ml. of 2:6-diiodophenol did cause some depression in oxygen consumption, and the rate of glycolysis increased to 238% of the control. 3:5-Diiodo-4-hydroxyphenylacetic acid had little effect on glycolysis at 50 and 100 μg/ml. At a concentration of 250 μg/ml. the rate was 166% of the control. Diiodotyrosine and its N-propionyl derivative were inactive in this test at concentrations as high as 200 μg/ml. and 100 μg/ml. respectively. 3:5 Diiodo-4-hydroxyphenyl acetic acid and diiodotyrosine did however depress oxygen consumption to some extent.

Effect of Triac on anaerobic glycolysis. In order to see if the stimulatory effect of Triac on aerobic glycolysis could be separated from the depression of oxygen uptake, the substance was added to intact cells under anaerobic conditions. Concentrations from 1×10^{-7} to 1×10^{-5} M were inactive anaerobically. Higher concentrations, which were stimulatory aerobically, were now inhibitory (Fig. 2). A similar result was obtained in another experiment in which the Triac effect on aerobic glycolysis was examined in the presence of .01 M azide.

Triac effects in cell fractions. When tested in a cell free homogenate, Triac exerted an inhibitory effect on both aerobic and anaerobic glycolysis (Table 3). Oxygen uptake and glucose utilization were also depressed. It

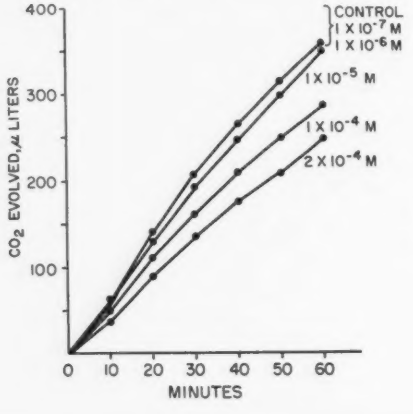

FIG. 2. *Effect of Triac on the anaerobic glycolysis of Ehrlich ascites tumor cells.* The experimental conditions were the same as in Figure 1 except that the gas phase was 95% N_2–5% CO_2. 1 ml of a 7% suspension of cells was used in this experiment.

TABLE 3. THE EFFECT OF TRIAC ON VARIOUS FRACTIONS OF ASCITES TUMOR CELLS

The fractions were prepared as described in the methods. The supernatant fraction and mitochondria were not obtained from the same batch of cells as the homogenate. The correspondence between the aerobic glycolysis of the homogenate on the first line and the reconstituted homogenate on the last line is coincidental. Each Warburg vessel contained 1 μ mole of ATP and 1 μ mole of DPN. The manometric procedures were carried out as described in Figure 1. Temperature, 28°. The CO_2 evolution was followed for 40 minutes in the case of the homogenate and 50 minutes in the experiments with supernatant fraction and mitochondria.

Cell fraction	Experimental conditions	Triac concentration		CO_2 production μ Liters	O_2 uptake μ Liters	Glucose utilization μ Moles
		μg/ml.	Molarity			
Homogenate	Aerobic	0		207	42	6.6
		6	1×10^{-5}	161	39	5.2
		62	1×10^{-4}	134	17	4.9
		124	2×10^{-4}	115	7	4.6
Homogenate	Anaerobic	0		198		6.7
		6	1×10^{-5}	153		5.4
		62	1×10^{-4}	113		5.1
		124	2×10^{-4}	87		4.3
Supernatant	Aerobic	0		118		
		6	1×10^{-5}	99		
		62	1×10^{-4}	41		
		124	2×10^{-4}	17		
Mitochondria	Aerobic	0		0		
Reconstituted Homogenate (Supernatant +mitochondria)	Aerobic	0		206		
		6	1×10^{-5}	193		
		62	1×10^{-4}	124		
		124	2×10^{-4}	72		

should be noted that the rate of glycolysis in ascites cell homogenate is not affected by the presence of oxygen (7). Aerobic glycolysis was similarly inhibited by Triac in a mitochondrion free supernatant fraction and in a homogenate reconstituted from supernatant fraction plus mitochondria. It can be seen that the addition of mitochondria to the supernatant fraction greatly increased the rate of glycolysis. This was presumably due to the addition of hexokinase which is localized in the particulate fraction to a large extent (7).

DISCUSSION

A stimulation of aerobic glycolysis may arise from interference with cellular metabolism at a variety of sites. It is therefore possible that the present group of active compounds may not have common points of action although their activities appear qualitatively similar. Any conclusions, therefore, regarding the relationship of structure to activity must be tentative. If the assumption is made, however, that these compounds do indeed have common points of action the following generalizations may be suggested.

Iodine in the phenyl rings gives rise to more activity than bromine. Three or four iodine substituents in the thyroxine analogues are required for maximal stimulation of aerobic glycolysis.

The most active stimulant of aerobic glycolysis is 2:6-diiodophenol. This suggests that the principal activity resides in this moiety of the other compounds tested. Substitution of groups in the 4 position of diiodophenol reduces its activity. When this substitution involves a grouping similar to the parent phenol, as in the compounds tetraiodothyropropionic acid and tetraiodothyroacetic acid, the reduction in activity is minimal. Perhaps this is because the substituting group retains some diiodophenol-like activity. This is suggested by the fact that 3:5-diiodo-4-hydroxyphenylacetic acid, which is the substituted group on tetraiodothyroacetic acid, has activity of its own. It would appear, however, that when the substituting phenolic constituent contains an amino or carbonyl group as in thyroxine, N-propionylthroxine and tetraiodothyropyruvic acid, activity is almost completely lost. The same pattern is observed when an aliphatic side chain is substituted directly on the 2:6 diiodophenol, i.e., diiodotyrosine and N-propionyl-diiodotyrosine.

With regard to the mechanism of the Triac effects, the study of broken cell preparations indicates that the compound may have at least 2 points of action since it inhibits both the glycolytic pathway and mitochondrial oxidation. What step or steps in glycolysis are blocked has not been determined except that these are below the phosphofructokinase level since inhibition is observed with fructose-1,6-diphosphate as substrate. Wolff and Wolff (8) have found an inhibitory effect of Triac on soluble dehydrogenases suggesting that the triosephosphate and lactic dehydrogenase reactions may be involved. With regard to inhibition of oxygen uptake in the mitochondria, it would be expected that this activity would be associated with some inhibition of oxidative phosphorylation, since this has been demonstrated in mitochondria of other tissues (9, 10).

It appears possible to account for the stimulation of aerobic glycolysis by the analogues in the intact cells as follows. The compounds may be postulated to have a preponderant activity on the mitochondrial system, which by inhibition of oxygen uptake gives rise to increased glycolysis by mechanisms involved in the well known Pasteur Effect. These mechanisms have recently been discussed by Chance (11), Lynen *et al.* (11), and Racker and Wu (11). This increase in glycolysis occurs despite concurrent inhibition of some step or steps in the glycolytic pathway. This latent inhibition can be shown by raising the Triac concentration until it eventually inhibits glycolysis even under aerobic conditions. The inhibition is also revealed by incubation of whole cells under anaerobic conditions were the only effect of Triac is to reduce slightly the production of CO_2. The greater effectiveness of diiodophenol as a stimulant of glycolysis may depend on its particular potency as an uncoupler of oxidative phosphorylation or on its lesser potency as an inhibitor of the glycolytic pathway. The effects of diiodophenol resemble those of dinitrophenol which produces the greatest stimulation observed thus far (1). Thus the lack of a direct relationship between

stimulation of aerobic glycolysis and inhibition of oxygen consumption can be readily understood in all cases if the final effect on glycolysis is considered as the resultant of effects on two pathways.

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AN ENDOCRINE MECHANISM INVOLVED IN TESTICULAR CHANGES PRODUCED BY A DL-ETHIONINE SUPPLEMENTED DIET: A STUDY OF MORPHOLOGIC CHANGES AND ENDOCRINE DYSFUNCTION[1]

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ABSTRACT

The endocrine-morphological relationship in testicular lesions of rats treated with ethionine and testosterone was investigated. This was done through indirect evaluation of the androgenic activity by the estimation of urinary 17-KS excretion.

It has been found that small and large doses of testosterone (12 days of treatment) prevent histologic testicular changes due to ethionine. However small doses of testosterone given over a longer period (24 days) did not prevent ethionine-induced testicular histologic changes. Large doses of testosterone did not prevent damage to spermatogenesis during continuous ethionine administration, but did prevent Leydig cell hyperplasia. Ethionine alone stopped spermatogenesis, and induced Leydig cell hyperplasia. Leydig cell hyperplasia therefore may have been compensatory.

Ethionine treatment decreased the excretion of 17-KS prior to the appearance of any testicular morphological changes. Therefore ethionine presumably affected the androgenic activity directly if 17-KS excretion may be used as an index of androgenic activity. Intraperitoneal (I.P.) administration of ethionine diminished the 17-KS excretion by two thirds. Since I.P. ethionine diminished in 12 days the excretion of 17-KS in the absence of histologic testicular changes and was followed by an increase in the excretion of 17-KS associated with Leydig cell hyperplasia after 24 days, this would suggest that functional changes precede histologic changes.

Morphological liver changes were demonstrable within 24 days after initiating oral ethionine therapy but not after I.P. ethionine. Gastrointestinal versus systemic absorption of the administered ethionine may be a factor.

Finally, it may be concluded that an endocrine-morphological relationship exists in the pathogenetic effect of ethionine on testicular changes observed in albino rats.

IN 1948 Hardwick (1) demonstrated a lipotropic action in the liver of female rats injected with *dl*-ethionine comparable to the action of

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diethyl choline. This was corroborated by many authors (2–6), some of whom stressed the fact that fatty infiltration of the liver occurred only in female rats. A sex difference in the nephrotoxic action of ethionine was also noted. It was observed that more kidney damage was produced in the male than in the female rats (7).

In 1952, it was reported (6, 8) that after male rats were castrated and treated with ethionine they developed fatty livers, whereas those that were castrated and treated with pellets of tesosterone did not develop these changes. Thus for the first time an endocrine factor was shown to be involved. More recent studies (9, 10) of testicular damage in albino rats led to the assumption that ethionine action may be directly upon the testis. The action of ethionine was thought to be comparable to that reported in many studies on the influence of diets deficient in essential amino acids (phenylalanine, theronine, histidine, leucine, tryptophane and methionine).

It has been found repeatedly that ethionine has a damaging effect on the parenchyma of the liver, beginning, in female rats and castrated males, with fatty infiltration and terminating in an extensive fibrosis (11) (intralobular hepatic type), bile duct proliferations (12), and the formation of hepatomas (13) and carcinomas (14). The interrelated changes in the liver and the testis already have been treated thoroughly in the literature (15, 16). Moreover, certain sex differences in regard to changes induced in the livers of rats submitted to ethionine treatment, have also been noted recently (17, 18).

Previous morphological studies have demonstrated that the damaging effect of ethionine on the testicles of rats (19, 20) may be due to an endocrine mechanism. The question now arises whether the changes noted in the testicles represented a direct ethionine action on sources of androgen production with subsequent morphologic changes in the testicle, or whether the changes in the testicles were secondary to estrogen increase due to lack of estrogen inactivation by an ethionine damaged liver.

It is within the scope of this study to report only the endocrinomorphological relationship in ethionine treated male rats through the evaluation of two parameters: (1) testicular histology and (2) androgen production as indicated by the urinary excretion of 17-ketosteroids (17-KS).

MATERIAL AND METHOD

Albino male rats (Sprague-Dawley) were used. Each rat weighed approximately 120 gm. at the beginning of the experiment. The rats were fed *ad libitum* on a diet of Purina lab chow except for one restricted diet group. Testosterone was used as a control to check on the rate of 17-KS excretion of the animals as well as its antagonistic effects to *dl*-ethionine.[3] Nine groups of rats, each group comprising six animals, and one group comprising three animals, were used. These groups were treated as shown in Table 1.

Ethionine was administered in two modes: either orally in 0.5% drinking water solu-

[3] Nutritional Biochemicals Corporation, Cleveland, Ohio.

TABLE 1

Material and Method					Histological Findings					
Mode of Administration					Effect on Leydig cell hyperplasia and tubular epithelium damage					
					Testicle—12th day		Testicle—24th day		Liver	Pancreas
Group	Number of rats	Ethionine	Testosterone	Cotton seed oil	Leydig cell	Tubular epithelium	Leydig cell	Tubular epithelium		
A	6	—	—	—	+	+	+	+	Normal	Normal
B	6	Oral 0.5% in drinking water	—	—	++	+−	+++	−	Mild oval cell proliferation	Severe acinar atrophy
C	6	—	0.5 Mg. IP 2x/week	—	+	+	+	+	Normal	Normal
D	6	Oral 0.5% in drinking water	0.5 mg. IP 2x/week	—	+	+	++	+−	Mild oval cell proliferation	Acinar atrophy
E	6	Oral 0.5% in drinking water	—	0.5 cc. IP 2x/week	+	+−	++	−	Mild oval cell proliferation	Severe acinar atrophy
F	6	—	—	0.5 cc. IP 2x/week	+	+	+	+	Normal	Normal
G	6	50 Mg. IP daily	—	—	+	+	+++	−	Mild oval cell proliferation	Severe acinar atrophy
H	6	Oral 0.5% in drinking water	25 Mg. IP 2x/week	—	+	+	+	−	Mild oval cell proliferation	Severe acinar atrophy
I	6	—	25 Mg. IP 2x/week	—	+	+	+	+	Normal	Normal
J	3	—	50 Mg. daily 6 days	—	Not evaluated				Not evaluated	

tion, or in an intraperitoneal 50 mg. daily injection. Testosterone[4] was injected intraperitoneally twice weekly in a dosage of 0.5 mg. or 25 mg. In order to check on the testosterone vehicle, cotton seed oil,[4] two groups were used as controls. Urine was pooled daily from each of the groups for evaluation of 17-KS according to the Holtorff-Koch (21) modification of the Zimmerman reaction. According to the technic used, androsterone gave a recovery of 85–95%.

On the 12th day of the experiment, a unilateral orchiectomy was performed on all the groups. The animals were sacrificed on the 24th day and the tissues, fixed in formalin, were submitted to histological processing and morphological evaluation. The morphological findings in the testicles were evaluated by a scheme (Fig. 1) in which a normal seminiferous tubular epithelium was graded (+), a deficient seminiferous epithelium (+−), and a totally destroyed seminiferous tubular epithelium (−). The degree of hyperplasia of Leydig cells in the interstitial tissues of the testicle was graded between (+) to (++++). Efforts were made ot relate testicular morphological changes to androgenic activity by the estimation of 17-KS in the first 12 days of the experiment and the last 12 days of the experiment. The morphology of the testicles on the 12th and 24th day were considered to correspond to the summation of experimental conditions existing during the 1st to the 12th day, and the 13th to the 24th day.

RESULTS

Morphological Testicular Changes and Their Relation to 17-KS Excretion

No Leydig cell hyperplasia or tubular epithelium damaging effect could be demonstrated in the untreated controls (Group A, Table 1) on either the 12th or the 24th day. The liver and pancreas, also were intact and of nor-

[4] Testosterone propionate as well as its cotton seed oil vehicle was supplied through the courtesy of the Upjohn Company, Kalamazoo, Michigan.

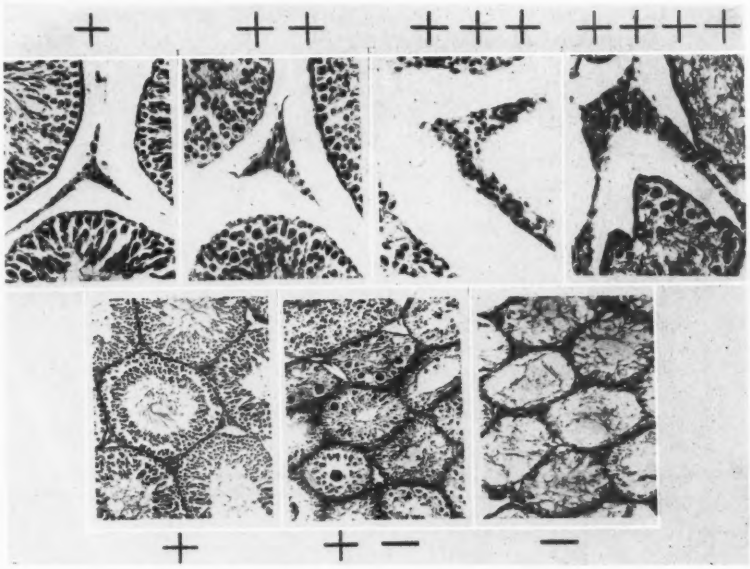

FIG. 1. Evaluative scheme for Leydig cells (upper) and seminiferous epithelium (lower).

mal pattern. Group B, (Table 1), receiving oral 0.5% ethionine demonstrated a Leydig cell hyperplasia grade (++) as a mean effect and a tubular epithelium damaging effect graded at (+−) on the 12th day. On the 24th day, Leydig cell hyperplasia had reached a mean effect of (+++) and at the same time the tubular epithelium was totally destroyed (Grade (−)).

Keeping these histological changes in mind the altered excretion of 17-KS in ethionine-treated rats (Curve B, Fig. 2) may be compared to that of the control group (Curve A, Fig. 2). One can note a decreased excretion of 17-KS throughout the ethionine experimental period. The administration of intraperitoneal ethionine (Group G) (Curve G, Fig. 2) produced an even greater effect on decreasing urinary 17-KS excretion in the first 12 days. However, intraperitoneal (assuring controlled administration) ethionine administration differed from the oral route in that there was a consistent rise in the excretion of 17-KS after the first 12 days although still less than the control mean daily output. When the corresponding histological changes (Group G, Table 1) were examined, very mild Leydig cell hyperplasia and tubular epithelium damage were apparent on the 12th day of the experiment. This corresponded to the time when the excretion of 17-KS was very low. However, during the second 12 days of the experiment when the 17-KS excretion rose, examination of the testicles at the end of this period (on the 24th day), showed a very severe Leydig cell hyperplasia and the tubular epithelium damage was at its maximum.

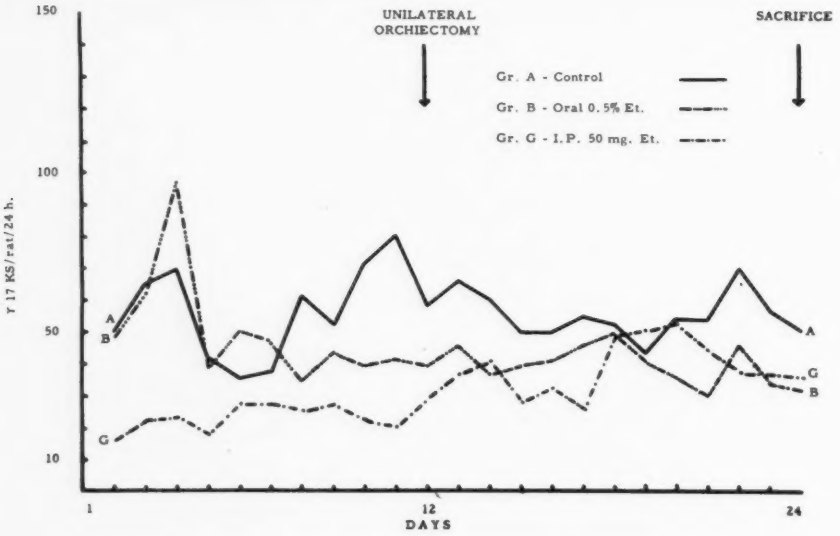

FIG. 2. 17-ketosteroid excretion in Groups A (control), B (oral ethionine) and G (intraperitoneal ethionine).

Furthermore there was no change in liver morphology after intraperitoneal ethionine administration, whereas changes were observed in the liver after oral ethionine (Group B, Table 1).

Testosterone Effects on Ethionine Induced Morphological Changes

Several experiments were undertaken in order to evaluate the effect of exogenous testosterone on ethionine-treated rats. Table 1 (Group D) demonstrates that no changes in the testicles were observed by the 12th day in rats receiving 0.5 mg. testosterone twice weekly. However, this protective effect on the 12th day did not persist to the 24th day when Leydig cell hyperplasia was present and the seminiferous tubular epithelium was damaged. When the dose of testosterone was increased to 25 mg. twice weekly (Group H, Table 1) the same protective effect on spermatogenesis as with the 0.5 mg. testosterone dosage (Group D, Table 1) was noted on the 12th day. Although there were severe changes in the seminiferous tubular epithelium on the 24th day, the Leydig cell hyperplasia was not apparent. The rate of excretion of 17-KS in these two groups can be compared in Figure 3. Curve D represents the rate of 17-KS excretion after a dose of 0.5 mg. of testosterone administered twice weekly. During the first 12 days of the experiment the rate of 17-KS excretion was quite low, but during the second half of the experiment it started to rise. However, when 25 mg. of testosterone was administered twice weekly the rate of excretion of 17-KS was relatively even and uniform (Curve H).

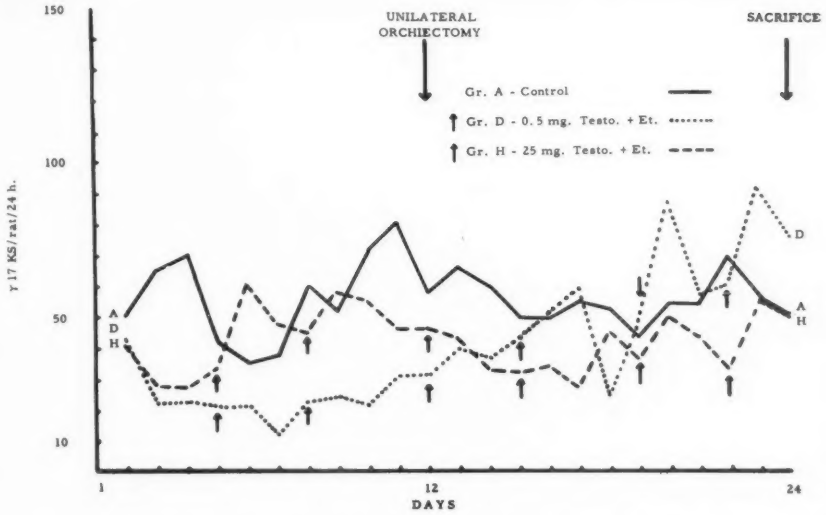

FIG. 3. 17-ketosteroid excretion in Groups A (Control), D (0.5 mg. testosterone plus ethionine) and H—(25 mg. testosterone plus ethionine).

Histologically, the Leydig cells demonstrated no hyperplasia at any time in this group (Group H, Table 1).

In contrast to the above it can be observed from the histological findings (Table 1) that the various control medications such as testosterone (Group C, and I) and cotton seed oil (Group F) did not exert any effect on the histology of the testicle when administered singly to rats. Furthermore, cotton seed oil did not exert any protective testicular effect in ethionine-treated rats (Group E).

Within the first 12 days of the study it can be noted from the mean 17-KS excretion values (Table 2) that the administration of ethionine orally (Groups B, E, H) was not as effective as I.P. administration in decreasing 17-KS excretion (Group G). The severe decline in the mean 17-KS excretion value seen in Group D during the first 12 days of the experiment

TABLE 2. MEAN VALUE γ 17/KS/24 HR./RAT

Group	Days	
	1–12	13–24
A. Control	58	53
B. Ethionine	45	39
C. Testosterone, 0.5 Mg.	71	44
D. Testosterone, 0.5 Mg. + Ethionine	27	57
E. 0.5 cc. Oil + Ethionine	42	44
F. 0.5 cc. Oil	44	36
G. Intraperitoneal Ethionine	25	39
H. Testosterone, 25 Mg. + Ethionine	44	39
I. Testosterone, 25 Mg.	38	48
J. Testosterone, 6x 50 Mg.	92	71

appeared due to an ethionine effect not accompanied by Leydig cell hyperplasia. This decline in 17-KS excretion was reversed in the second 12 days. Group J demonstrates the effect of 6 consecutive doses of 50 mg. testosterone each, on the rate of 17-KS excretion in the intact animals.

DISCUSSION

Testosterone propionate administered for 12 days to *dl*-ethionine-treated rats in a 0.5 mg. dose injected intraperitoneally, was found to counteract the damaging effect of *dl*-ethionine on the seminiferous epithelium. The establishment of this protective effect is further substantiated by the lack of hyperplasia of Leydig cells. Such hyperplasia is thought to be compensatory in the presence of damage to the seminiferous epithelium. However, in view of the fact that there was a severe decline of 17-KS excretion, despite the absence of morphological testicular changes on the 12th day, one can conclude: (1) that ethionine has a direct effect on androgen production, and (2) that functional changes may precede morphological changes.

When testosterone propionate was administered intraperitoneally to *dl*-ethionine treated rats, in a dose of 25 mg. daily (Group H) it also had a total protective effect on the seminiferous epithelium on the 12th day with no hyperplasia of Leydig cells. However, the fact that the 17-KS were not depressed, would suggest that the exogenous testosterone was administered in sufficient amounts (25 mg.) to counteract the ethionine depressive effect on 17-KS excretion observed in Group D where only 0.5 mg. testosterone was administered. This is further substantiated by continued lack of Leydig cell hyperplasia in this group (H) on the 24th day. That the seminiferous tubular epithelium was totally destroyed on the 24th day despite a 25 mg. dose of exogenous testosterone, suggests that the exogenous testosterone had only a limited protective effect.

When testosterone was administered alone for 24 days in a dose of 0.5 or 25 mg. to normal rats, intraperitoneally twice weekly, spermatogenesis and the pattern of Leydig cells were not affected adversely. The larger dose of testosterone appeared to be of sufficient quantity to maintain an even level of daily 17-KS excretion during the 24 days of the experiment without any morphological manifestations interpretable as a suppressive effect due to excess testosterone. It is unlikely therefore that the same dose (25 mg.) of testosterone administered to ethionine-treated rats is a contributing factor in producing damage of the seminiferous epithelium. Therefore when this epithelium is damaged in ethionine-treated rats, the effect can be attributed solely to ethionine.

The fact that no increase in 17-KS excretion was noted in normal rats despite the administration of 25 mg. testosterone, suggests that exogenous testosterone, at this dose, does have a suppressive effect on the Leydig cells. Moreover, the Leydig cell hyperplasia, expected in ethionine-treated rats, did not appear in the presence of a sufficient amount of exogenous testosterone.

It appears that androgen deficiency precedes morphological testicular damage, whether the ethionine is given orally or intraperitoneally (cf Groups D and H). It could be observed that a depression in the rate of excretion of 17-KS does not necessarily stimulate a simultaneous hyperplasia of Leydig cell. There seems to be a latent period between the depression of 17-KS excretion and the appearance of damage in the tubular epithelium. It has been demonstrated that the hyperplasia of Leydig cells is of a compensatory nature and follows a depression in the rate of 17-KS excretion after a latent period which corresponds to that of the appearance of injury to the tubular epithelium. Hyperplasia of Leydig cells does not appear in the presence of a sufficient quantity of exogenous testosterone despite a progressive damage to the tubular epithelium.

The fact that the administration of a large quantity of exogenous testosterone to ethionine-treated rats immediately increased the rate of excretion of 17-KS, suggests that the system for the transport of androgens or their metabolites in the organism is intact. This suggests that the ethionine-treated animal can metabolize androgen, and that the deficiency of 17-KS excretion is due to a deficiency in the production of some 17-KS precursor. It still remains to be seen whether the source of the 17-KS precursor is testicular or extratesticular in origin.

Finally, it may be concluded that an endocrine morphological relationship exists in the pathogenetic effect of ethionine on testicular changes in albino rats. The decline in the excretion of 17-KS is in favor of hypoandrogenism.

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THYROTROPIN-INDUCED RELEASE OF IODIDE FROM THE THYROID[1]

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ABSTRACT

The early effects of thyrotropin on the iodine metabolism of the dog thyroid were studied by measuring plasma concentrations of stable and radioactive iodine in arterial and thyroid venous blood, 2 to 4 hours after I^{131} administration. When organic binding of iodide was not prevented, TSH administration led to the prompt release from the gland of TCA-soluble as well as TCA-precipitable stable iodine with relatively little change in the glandular clearance of circulating labeled iodide; iodide release was considerably enhanced by perchlorate. When organic binding was blocked (methimazole) TSH led to the release of both stable and radioactive iodide from the gland and the released iodide was of lower specific activity than arterial iodide. The data suggest that TSH enhances the formation of iodide in the gland, probably by deiodination of organic iodine compounds (iodotyrosines). The iodide so produced seemed to be at least partially exchangeable with circulating iodide concentrated by the gland when organic binding was blocked, but when binding was permitted there was little evidence of such exchange during a relatively short period of observation. When organic binding was not prevented, the administration of perchlorate to both TSH-injected and control animals led to glandular release of iodide of specific activity considerably less than that of circulating iodide, suggesting the presence within the thyroid of iodide poorly exchangeable with iodide newly entered from the circulation.

STUDIES using the technique of thyroid venous cannulation in the dog have shown that the administration of thyrotropin (TSH) may lead to the prompt release from the thyroid not only of organic iodine but also of inorganic iodide (1, 2). The dog thyroid apparently contains little inorganic iodide that has newly entered from the circulation (3); since the release of labelled iodide from the gland induced by TSH was most apparent when administration of radioactive iodine several days earlier had led to appreciable labelling of glandular iodine and especially when the iodide-concentrating mechanism of the gland had been inhibited by perchlorate, it appeared that TSH stimulated the formation within the gland of inorganic iodide by deiodination of organic iodine compounds, and that the iodide

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released from the gland was derived from this source rather than from iodide newly "trapped" from the circulation (1). It seemed of interest to study further the iodide-releasing effect of TSH under conditions in which the specific activities of circulating iodide and of glandular iodine were widely disparate, iodide derived from deiodination of organic iodine stores being relatively poorly labelled in comparison to the circulating iodide, and also to study the release of iodide when organic binding of iodine was prevented as well as when it was not.

METHODS

Experiments were done using adult dogs of either sex, each animal being used for one experiment. The details of the surgical, analytic and other procedures used have been previously described (1). The experimental observations were made in animals anesthetized with pentobarbital; one lobe of the thyroid was removed and from a cannula inserted into the inferior thyroid vein of the remaining lobe samples of thyroid venous blood were obtained consecutively for a period of 1 to 2 hours. The time required to obtain each sample (approximately 5 ml.) was recorded, and this permitted an estimate of blood flow. Arterial blood samples were obtained repeatedly from the femoral artery and test substances were injected into the femoral vein. Blood samples were obtained beginning 2 to 3 hours after the intravenous injection of I^{131} (50 μc, Squibb oral therapeutic solution of sodium radioiodide). The concentration of stable and of radioactive iodine was measured in the separated plasma of arterial and thyroid venous blood. In preliminary experiments only a small fraction (less than 5%) of the total I^{131} was found to be precipitable with trichloroacetic acid (TCA) in specimens of arterial and thyroid venous plasma obtained 2 to 4 hours after the tracer dose had been given; hence, the radioactivity of whole plasma alone was determined, and this was considered to represent the concentration of inorganic iodide-I^{131}. The arterial and thyroid venous plasma concentration of total and TCA-precipitable stable iodine were determined (4, 5) and the difference between these values was considered to be the concentration of stable inorganic iodide.

Three groups of experiments were done. A) *Organic binding permitted* (16 dogs). The dogs received I^{131} approximately 2 hours before the beginning of the blood sampling period. In 11 experiments the procedure was to obtain 2 or 3 samples of thyroid venous and arterial blood during a period of 20 to 30 minutes (the control period), and then TSH (Thytropar, Armour), 5 or 10 USP units, was given intravenously, and samples of blood were collected for the next 45 to 60 minutes, at which time perchlorate (100 mg. of the sodium or potassium salt) was injected intravenously, and blood samples were collected for 20–40 minutes longer. In three experiments saline was given instead of TSH, and in the remaining 2 experiments the procedure was modified in that perchlorate was given after the initial control period and 30 minutes before TSH was given.

B) *Organic binding blocked* (15 dogs). Methimazole[2] in a dosage of 20–30 mg. was given intravenously one-half hour before I^{131} was injected, and $2\frac{1}{2}$ hours before the start of the blood sampling period. In 7 experiments (group B-1) after the initial control sampling period, TSH (5–10 U) was given, and 40 to 70 minutes later perchlorate (100 mg. of the sodium or potassium salt) was injected intravenously and collection of blood samples continued for 30 minutes longer. In 6 experiments (group B-2) sodium perchlorate was given after the initial control period, 100 mg. in 3 cases, 10 mg. in one,

[2] Generously supplied by the Eli Lilly Company through the courtesy of Dr. Ewald Rohrmann.

and 2 mg. in two cases. In two experiments, (group B-3) TSH (10 U.) was given intramuscularly 22 hours before the sampling period and 18 hours before the methimazole and I^{131} were injected, and perchlorate was given after the initial control period of blood sampling.

C) *Glandular iodine stores pre-labelled* (2 dogs). These studies were similar to those previously performed (1) in that I^{131} was given 2 days (dog C-1) and 4 days (dog C-2) before the blood sampling period in order to label glandular iodine stores. In both dogs thyrotropin was given after the control sampling period; in dog C-1 perchlorate had been given 30 minutes before operation and in dog C-2 perchlorate was given 1 hour after TSH. Thyroid venous and arterial plasma concentration of total and TCA-precipitable I^{131} and I^{127} were determined. In addition, the thyroid gland of dog C-2 was removed after the experiment, ground in saline and incubated with pancreatin for 2 days (6). The butanol extract of the acidified digest was extracted with the Blau reagent (7), to achieve separation of thyroxine from iodotyrosines, and the concentration of I^{127} and I^{131} in the organic and aqueous phases was determined.

RESULTS

Group A experiments. During the control period of each of the 16 experiments the plasma concentration of I^{131} was higher in arterial than in thyroid venous blood, and the average value for thyroid clearance of circulating I^{131} obtained from the product of the extraction ratio (A–V)/A and measured blood flow was 0.19 ml/min. and the average value for the extraction ratio was 0.3. The concentrations of stable iodide in thyroid venous and arterial plasma were nearly the same in the control period, while the venous PBI^{127} tended to be slightly higher than the arterial value; thus in arterial plasma, TCA-precipitable I^{127} averaged 1.7 μg/100 ml. (range 0.2–3.4), and TCA-soluble I^{127} averaged 2.9 μg/100 ml. (range 0.8–5.1), while in thyroid venous plasma, the average PBI^{127} value was 2.9 (range 0.8–7.3) and TCA-soluble I^{127} was 2.9 (range 0.6–6.4). Initial concentration differences between arterial and thyroid venous plasma in individual experiments seldom exceeded 1–2 μg/100 ml. (Table 1).

In the 11 experiments in group A in which the early effects of TSH were studied there appeared to be some tendency for the thyroid clearance[3] of circulating I^{131} to decrease during the hour after the injection of TSH. The change in thyroid clearance between control and TSH periods was neither large nor consistent; in 4 experiments clearance remained the same and in 7 it decreased, the average value in the TSH period being 0.14 ml/min. as compared with 0.17 ml/min. in the control period (Table 1). In most experiments there was some decrease in the extraction ratio during the experimental period. Although a positive arterio-thyroid venous (A–V) concentration difference for I^{131} persisted throughout the TSH period in

[3] Thyroid "clearance" of circulating iodide was calculated from the average value of the product of the extraction ratio for radioiodide and the measured thyroid venous blood flow of samples drawn in the control period (before TSH injection) and in the period between TSH injection and the administration of perchlorate. No correction was made for hematocrit which was usually about 40%.

TABLE 1. EFFECT OF TSH UPON GLANDULAR RADIOIODIDE CLEARANCE AND STABLE IODINE RELEASE (GROUP A EXPERIMENTS)

Experiment number	Material injected	Duration of sampling period after injection (min.)	Average extraction ratio I^{131} (A-V)/A		Average thyroid venous blood flow (ml./min.)		Thyroid "clearance," I^{131} (ml./min.)		Plasma stable iodine (μg/100 ml.)							
									PBI				TCA-soluble I			
									Preinjection		End of post-injection period		Preinjection		End of post-injection period	
			Pre-injection	Post-injection	Pre-injection	Post-injection	Pre-injection	Post-injection	A*	V*	A	V	A	V	A	V
1	TSH	41	0.43	0.41	0.49	0.37	0.21	0.15	0.2	0.8	0.2	2.1	2.7	3.4	2.8	13.9
2	TSH	69	0.098	0.092	1.11	1.05	0.093	0.095	1.8	1.8	1.7	7.3	5.1	6.4	7.0	9.9
3	TSH	79	0.23	0.26	0.345	0.20	0.078	0.059	1.6	3.5	2.9	6.4	2.0	1.3	1.5	5.6
4	TSH	53	0.27	0.18	0.70	0.74	0.19	0.14	1.6	3.7	1.7	10.0	0.8	1.6	0.9	4.8
5	TSH	61	0.41	0.37	0.83	0.99	0.33	0.35	1.7	1.0	1.3	10.3	3.1	3.5	3.9	6.2
6	TSH	73	0.31	0.29	0.58	0.48	0.18	0.13	3.4	3.8	3.5	26.0	2.0	3.6	2.3	4.3
7	TSH	50	0.46	0.30	0.19	0.28	0.09	0.08	2.3	3.4	1.9	8.6	2.5	1.2	1.7	2.1
8	TSH	62	0.39	0.23	0.87	0.58	0.30	0.18	1.6	1.6	3.4	4.3	1.8	0.6	0.6	0.9
9	TSH	65	0.38	0.33	0.59	0.70	0.23	0.23	1.9	3.3	1.4	8.0	3.0	2.6	4.2	5.3
10	TSH	66	0.30	0.25	0.34	0.33	0.10	0.078	1.1	2.4	1.6	8.4	2.4	2.7	1.8	10.9
11	TSH	53	0.26	0.17	0.40	0.44	0.10	0.075	1.5	2.6	1.4	7.9	1.4	1.4	1.2	7.8
Average			0.32	0.26	0.59	0.56	0.17	0.14	1.7	2.5	1.9	9.0	2.4	2.5	2.5	6.5
12	saline	55	0.19	0.22	1.03	0.88	0.20	0.19	1.9	3.5	1.8	4.2	3.7	4.9	3.8	5.0
13	saline	47	0.23	0.16	0.96	0.72	0.22	0.12	1.2	3.7	1.2	4.0	4.7	4.5	4.0	4.0
14	saline	42	0.42	0.45	0.48	0.42	0.20	0.20	1.7	7.3	1.6	7.2	3.6	4.4	4.2	4.0
Average			0.28	0.28	0.82	0.67	0.21	0.17	1.6	4.8	1.5	5.1	4.0	4.6	4.0	4.3
15	none	0	0.35		1.0		0.35		1.4	1.3			3.5	2.7		
16	none	0	0.28		0.7		0.20		2.5	2.8			3.3	3.1		

* A and V refer, respectively, to arterial and thyroid venous plasma.

each experiment, in 8 of the 11 experiments the thyroid venous plasma concentration of TCA-soluble I^{127} rose by 2.7 to 11.5 μg/100 ml. in this period and in 9 cases the value exceeded by at least 1 μg/100 ml. the simultaneous arterial concentration, indicating the net loss of iodide from the gland (Table 1). Thus, while the average values for arterial plasma concentration of TCA-soluble iodine in these experiments was 2.4 μg/100 ml. before TSH and 2.5 μg/100 ml. at the end of the TSH period, the corresponding values in thyroid venous plasma were 2.5 and 6.5 respectively. The thyroid venous plasma PBI^{127} concentration also rose after TSH injection, from an average value of 2.5 μg/100 ml. in the control period to the average value 9.0 μg/100 ml. at the end of the TSH period (Table 1).

Injection of perchlorate approximately 1 hour after TSH injection resulted in the abrupt abolition of the positive A–V difference in I^{131}, and after a transient period during which venous I^{131} exceeded arterial, the concentration of arterial and thyroid venous plasma I^{131} usually became nearly equal. However, the concentration of stable iodide in thyroidal venous blood continued to be appreciably greater than the arterial value throughout the period of observation (11–45 minutes) after perchlorate injection. The striking difference in these experiments (group A, Experiments 1, 2, 4–11) between glandular release of radioiodide and of stable iodide following the administration of perchlorate is shown in Table 2. The specific activity of the iodide released from the thyroid within the first few minutes after perchlorate was only 5.3 percent of the concurrent arterial

TABLE 2. EFFECT OF PERCHLORATE UPON THYROID DISCHARGE OF STABLE IODIDE AND I^{131} IN TSH-TREATED AND CONTROL DOGS (GROUP A EXPERIMENTS)

Experiment	TSH pretreatment	Duration of post-perchlorate period (min.)	Specific activity of plasma iodide post-perchlorate* (% dose I^{131}/μg I)			Iodide released in post-perchlorate period		Accumulation time equivalent of iodide released in post-perchlorate period (min.)†	
			A	V	(V-A)	Stable iodide (μg)	I^{131} (% dose)	$t_{I^{127}}$	$t_{I^{131}}$
1	+	36	0.32	0.057	0.014	2.26	0.0124	398	5.1
2	+	19	0.13	0.073	0.00	0.86	0.000	149	0.0
3‡	+	0							
4	+	28	0.76	0.086	0.019	0.64	0.0073	420	6.5
5	+	11	0.14	0.076	0.029	0.79	−0.0016	77	0.0
6	+	24	0.52	0.31	0.013	0.74	0.008	187	5.0
7	+	24	0.30	0.085	0.026	0.75	0.018	333	39
8	+	45	0.68	0.24	0.031	0.22	0.0028	56	1.6
9	+	23	0.13	0.056	0.0063	1.18	0.0036	161	2.8
10	+	30	0.54	0.12	0.016	0.63	0.0043	299	3.9
11	+	28	0.93	0.091	0.013	1.57	0.0102	1300	8.3
Average		29						338	7.2
12	0	20	0.20	0.081	0.0043	0.92	0.0032	120	1.9
13	0	34	0.15	0.013	0.00073	2.75	0.0046	292	3.0
14	0	32	0.15	0.11	0.050	0.44	0.0026	61	1.9
15	0	25	0.20	0.19	0.14	−0.07	−0.0044	0	0
16	0	33	0.13	0.16	—§	0.00	0.0097	0	7.9
Average		29						95	2.9

* A and V represent the specific activity of arterial and thyroid venous plasma iodide, respectively, at that time after perchlorate injection at which the arterio-venous I^{131} concentration difference was first observed to be negative; V-A denotes the specific activity of the venous iodide increment at that time, and was calculated by dividing the veno-arterial concentration difference of I^{131} by that of TCA-soluble stable iodine.

† See text.

‡ No perchlorate was given in this experiment.

§ No stable iodide released.

plasma iodide value, and in the entire period of observation after perchlorate 0.96 μg (average) of stable iodide was released and 0.0065% of the dose of I^{131}. To obtain a better basis for comparison of stable and radioactive iodide release, the quantities released were expressed as accumulation-time equivalents for stable and radioactive iodide; i.e., the time that would have been required at observed values of initial glandular clearance of I^{131} and average arterial plasma concentrations (in the pre-perchlorate period) of stable and radioiodide, for the glandular accumulation of the quantities of stable iodide and of I^{131} released after perchlorate. The accumulation times were thus:

$t_I 127$ (min.) =

$$\frac{\text{Stable iodide released } (\mu g)}{\text{initial clearance } I^{131} \text{ (ml./min.)} \times \text{average arterial plasma stable iodide concentration } (\mu g/\text{ml.})}$$

and

$t_I 131$ (min.) =

$$\frac{I^{131} \text{ released (\% dose)}}{\text{initial clearance } I^{131} \times \text{average arterial plasma } I^{131} \text{ concentration (\% dose/ml.)}}$$

The average value for t_I^{127} was 338 minutes, and for t_I^{131} 7.2 minutes.

One of the group A experiments in which TSH was given is illustrated in Figure 1.

In the three control experiments (numbers 12–14, Table 1) in which

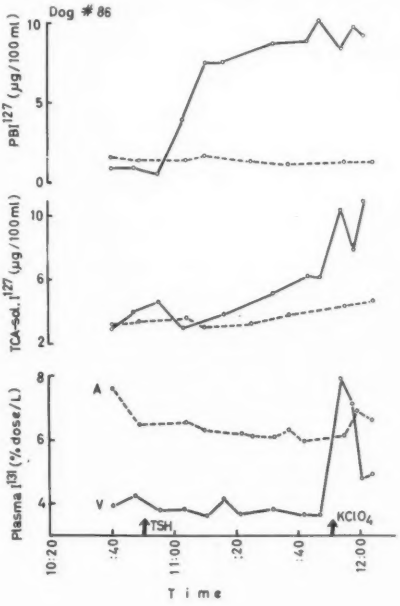

FIG. 1. Group A experiment. Injection of TSH (10 U.) 3 hours after I^{131} administration led to glandular release of stable PBI and iodide, with little change in arterio-thyroid venous I^{131} difference. The quantity of stable iodide and of I^{131} released from the gland in the 7 minute period immediately following perchlorate injection could have been accumulated by the gland in 45 minutes and in 4.5 minutes, respectively, in the control period. The decrease in thyroid venous plasma radioiodide concentration to values below the arterial values approximately 10 minutes after perchlorate injection in this experiment was unusual in this series and is unexplained. In this and subsequent figures, dotted lines represent arterial values; solid lines, thyroid venous values.

saline solution was injected instead of TSH, no appreciable changes in the plasma concentration of thyroid venous PBI^{127} or inorganic $iodide^{127}$ occurred before perchlorate was given; although the average thyroid clearance of plasma I^{131} decreased slightly during the period of observation from 0.21 to 0.17 ml./min., this was largely due to the appreciable reduction in clearance in Experiment 13 (Table 1). When perchlorate was given in these experiments and in two others (numbers 15 and 16), there was a transient release of I^{131} from the gland, the quantity released being equivalent to that which could have been accumulated by the gland in 2.9 minutes (average)

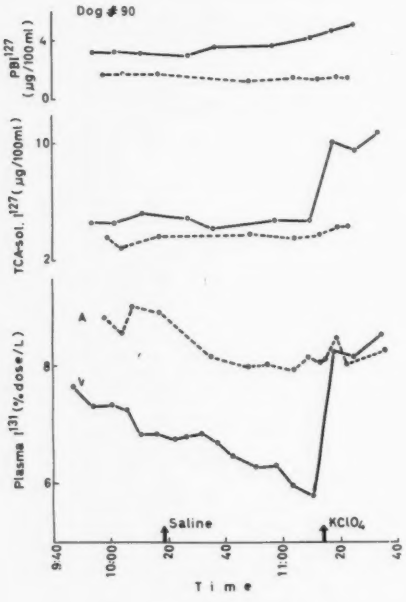

FIG. 2. Control experiment, group A. See text.

under the conditions of blood flow and extraction ratio recorded in the pre-perchlorate period. In 3 of the 5 control experiments perchlorate injection was followed by a considerable release of TCA-soluble stable iodide from the gland, comparable in degree to that observed in the majority of dogs that had received TSH, while in the remaining 2 experiments no appreciable discharge of stable iodide occurred. The average $t_{\frac{1}{2}}^{127}$ was 95 minutes. Since in those experiments in which stable iodide release occurred after perchlorate administration there was no corresponding change in thyroid venous PBI concentration, this suggested that net release of iodide from the gland was not necessarily dependent upon enhanced secretion of PBI. A control experiment is shown in Figure 2.

The differences between the control and TSH groups with regard to the mean accumulation time equivalents for stable and radioiodide released

after perchlorate (Table 2) were not statistically significant, for t_I^{127} the probability that the difference between the means was due to chance was $>.10$, while for t_I^{131} the p value was $>.30$.

In the 2 experiments in which perchlorate was given before TSH injection, the arterio-venous concentration differences for radioiodide and stable iodide were virtually zero at the time TSH was given. TSH injection resulted in generally parallel and progressive increases in concentration of venous stable iodide and of PBI, both being distinct approximately 20 minutes after the injection, while glandular clearance of plasma I^{131} remained negligible. Approximately 45 minutes after TSH had been given, the concentration of TCA-soluble stable iodine in thyroid venous blood had risen from 3.1 to 10.6 μg/100 ml. (concurrent PBI increase from 2.1 to 7.0 μg/100 ml.) in Experiment 15, while in Experiment 16 the corresponding values for iodide were 4.6 to 12.3, and for PBI 1.8 to 7.2 μg/100 ml. The data obtained in these experiments before and after perchlorate injection but before TSH injection are shown in Tables 1 and 2 (Experiments 15 and 16).

The absence of major changes in the glandular clearance of circulating iodide during the TSH period and the disparity between the small transient release of I^{131} from the gland induced by perchlorate as compared with the appreciable, sustained release of stable iodide suggested that the iodide-releasing effect of TSH did not predominantly involve iodide newly available to the gland from the circulation. This was consistent with the promptness and regularity with which TSH induced glandular iodide release in animals pre-treated with perchlorate, in the experiments described here as well as those reported earlier (1), when glandular accumulation of plasma iodide was minimal.

Group B experiments (*organic binding blocked*). Arterial and thyroid venous plasma concentrations of I^{131} were nearly equal in the initial control period (2 to 3 hours after methimazole and I^{131} administration) in the 13 experiments in groups B-1 and B-2, indicating that equilibrium between plasma and glandular iodide had probably been achieved.

Group B-1. (TSH injection 40 to 70 minutes before perchlorate administration). In 6 of the 7 experiments injection of TSH after collecting the initial samples of arterial and thyroid venous blood resulted in a prompt rise in the concentration of I^{131} in thyroid venous plasma so that it exceeded the arterial value; there was little change in blood flow. A representative experiment is shown in Figure 3. The rise in the thyroid venous plasma concentration of I^{131} was distinct in the first blood sample collected after TSH injection; since the time required to collect this sample averaged 13 minutes (range 8 to 20 minutes) it is probable that the increment might have been detected even sooner had samples been obtained more frequently. The venous I^{131} concentration rose promptly to a peak value which averaged 28% greater than the arterial value (range 10–50%), and then

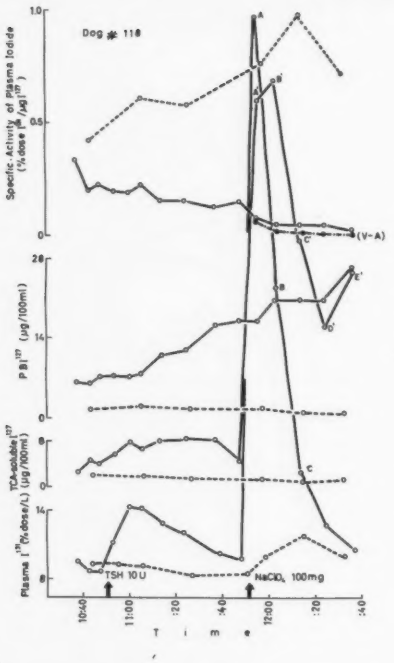

FIG. 3. Group B-1 experiment. Prompt glandular release of iodide when TSH was injected 2 hours after I^{131} administration to dog pre-treated with methimazole. Specific activity of thyroid venous iodide decreased after TSH: the iodide discharged after perchlorate injection was of progressively lower specific activity. The value of the points A, B and C on that portion of the curve describing the thyroid venous plasma concentration of I^{131} which extends beyond the indicated part of the abscissa scale were, respectively, 57.3, 33.4 and 17.3% of the dose of I^{131} per liter. The values of the determined points on that portion of the curve of thyroid venous plasma concentration of TCA-soluble iodine which extended beyond the abscissa scale were: A^1 72; B^1 75.5; C^1 43.3; D^1 29.8; and E^1 37.8 µg per 100 ml.

generally declined, approaching but always exceeding the arterial value. The thyroid venous concentration of TCA-soluble stable iodine usually rose toward the latter part of the hour following TSH injection so that it became greater than the arterial value, and the TCA-precipitable stable iodine concentration increased after TSH just as in the experiments of group A. The thyroid venous increment in PBI^{127} generally became distinct[4] somewhat after the increment in I^{131}, the average time being 28 minutes (range 21–47). Injection of perchlorate led to a prompt increase in the thyroid venous plasma concentration of both I^{131} and TCA-soluble I^{127}. The maximal value for the plasma concentration of I^{131} in thyroid venous blood was in the first sample collected after perchlorate administration, and in subsequent samples the value fell toward the arterial value. The increase in the venous concentration of stable iodide resulting from perchlorate tended to be more sustained. The specific activity of the iodide released from the gland after perchlorate was much lower than that of the arterial iodide; the specific activity of the released iodide was estimated from the ratio: veno-arterial concentration difference in I^{131} (% dose/l. /veno-arterial concentration difference in TCA-soluble stable iodine (µg/l.). The average value of the specific activity of the iodide released

[4] An increase in concentration was considered distinct if it was 1.0 µg/100 ml. or greater.

during the time required for collection of the first venous sample after perchlorate was 19.7% of the arterial value (Table 3); because of the sustained nature of the stable iodide release as compared with the transient

TABLE 3. SPECIFIC ACTIVITY OF IODIDE RELEASED FROM THYROID AFTER PERCHLORATE INJECTION IN TSH-TREATED AND CONTROL DOGS (GROUP B EXPERIMENTS)

Experiment	TSH dosage (USP units)	Interval between TSH and perchlorate injections (min.)	Post-perchlorate period			
			Interval after perchlorate injection (min.)	Specific activity (% dose $I^{131}/\mu gI$)		Relative specific activity B/A×100
				A arterial iodide	B released iodide	
Group B-1						
1	5	54	0–12	0.295	0.044	15.0
			12–25	0.302	0.0128	4.2
2	4	60	0–12	0.23	0.13	57.0
			12–22	0.23	0.0257	11.0
3	5	81	0–8	0.048	0.0093	19.4
			8–14	0.0695	0.0035	5.0
			15–21	0.076	0.00225	3.0
			21–28	0.073	0.00147	2.0
4	5	48	0–8	0.32	0.032	10.0
			9–14	0.30	0.029	9.7
			14–19	0.29	0.023	7.9
			19–24	0.31	0.014	4.5
5	5	58	0–12	0.22	0.0284	12.9
			12–23	0.23	0.0003	0.13
6	10	58	0– 4	0.208	0.071	34.0
			4– 6	0.238	0.045	19.0
			10–15	0.242	0.043	17.7
7	10	64	0– 7	0.76	0.067	8.8
			7–17	0.98	0.029	3.0
			18–17	0.98	0.013	1.3
			28–37	0.72	0.0088	1.2
			37–53	0.72	0.0016	2.2
Group B-2						
8	0	—	0–11	0.267	0.25	93.0
			20–32	0.30	0.027	9.0
9	0	—	0–14	0.49	0.106	22.0
			14–29	0.39	0.082	21.0
			30–41	0.37	0.072	19.0
10	0	—	0–11	0.22	0.22	100.0
			11–26	0.22	0.15	68.0
11	0	—	3– 8	0.28	0.57	203.0
Group B-3						
12	10	1320	0– 1	0.085	0.023	27.0
			1– 2	0.100	0.033	33.0
			3– 5	0.100	0.034	34.0
			5– 6	0.125	0.022	17.6
			9– 8	0.114	0.0324	28.0
13	10	1320	0– 3	0.075	0.030	40.0
			25–40	0.069	0.005	7.2
			50–60	0.084	0.006	7.1

character of the I^{131} discharge, the specific activity of the released iodide became much smaller in subsequent samples, and in some cases where there was almost no V-A concentration difference in I^{131}, specific activity could not be accurately determined.

Group B-2. In the 4 experiments in control group B-2 in which the methimazole pre-treated dogs were given enough perchlorate to inhibit completely the iodide-trapping mechanism (as judged by the zero (A-V) difference in I^{131} after the release of thyroidal I^{131}), the values for the specific activity of the iodide released in the early minutes after perchlorate administration were nearly equal to the arterial value in 2 cases, higher in one, and lower in one, the average value being 105% of the arterial value (Table 3). Thus the iodide discharged from the gland by perchlorate under conditions of inhibited organic binding of iodine was of considerably lower specific activity in the dogs which had received TSH 1 hour before the perchlorate than in the control animals. In the control group, as in the TSH group, the specific activity of the released iodide diminished with time after perchlorate because the release of stable iodide persisted longer than that of I^{131}.

In two of the experiments in group B-2, after the initial control period, a small quantity (2 mg.) of sodium perchlorate was given which produced only partial inhibition of the iodide-trapping mechanism; one such experiment is shown in Figure 4. The small dose of perchlorate led to a sudden release of I^{131} from the gland, but the thyroid venous I^{131} concentration then stabilized at a value well above the arterial value, and the thyroid/plasma I^{131} concentration gradient was presumably smaller than it had been in the control period. Injection of TSH then led to an immediate large increase in the thyroid venous plasma concentration of stable iodide and of I^{131}, following which the venous I^{131} concentration declined in exponential fashion similar to what occurred after a large dose of perchlorate, and the specific activity of thyroid venous iodide decreased progressively.

Group B-3. TSH was given intramuscularly to 2 dogs 22 hours before the blood sampling period, and 18 hours before administration of methimazole and I^{131}. In the initial blood sampling period, the expected high values for venous concentration of PBI^{127} were found, the values being 3 to 4 times greater than the corresponding arterial values, and the stable iodide concentration of thyroid venous blood was twice the arterial concentration, while the arterial and thyroid venous plasma concentration of I^{131} were nearly the same. Perchlorate injection led to the release from the gland of iodide of specific activity less than that of the arterial plasma iodide; the specific activity of the iodide first released (calculated from the data from the first sample of thyroid venous blood obtained after perchlorate) was in 1 case 27% and in the other case 40% of the concurrent arterial iodide specific activity (Table 3). Since the iodide released under these conditions

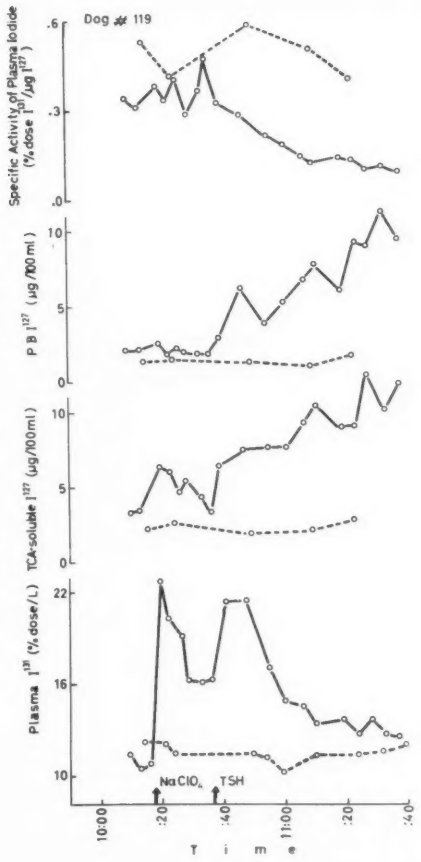

FIG. 4. Group B-2 experiment. Effect of TSH upon glandular iodide release after partial inhibition of iodide "trapping" mechanism by 2 mg. $NaClO_4$ given to dog pre-treated with methimazole. Specific activity of thyroid venous iodide decreased considerably following TSH injection. The thyroid/plasma I^{131} concentration ratio of the uncannulated lobe of the thyroid, removed just before the start of the collection period of thyroid venous blood from the contralateral lobe, was 43; the thyroid/plasma ratio of the lobe which had been cannulated was 3.1 at the end of the experimental period.

was of lower specific activity than the average for control experiments (B-2), these results suggested the possibility that glandular iodide release may persist for at least 24 hours after the administration of TSH.

Group C. In the 2 experiments in which the organic iodine stores of the gland had been labeled by giving I^{131} 2–4 days before the surgical procedures, TSH administration led to the prompt release from the gland of hormonal and TCA-soluble iodine, the increment of inorganic iodide being enhanced by perchlorate injection, in agreement with results previously reported (1). In one of these experiments (C-1), shown in Figure 5, the specific activity of the iodide released from the gland was nearly twice that of the PBI secreted and only one half that of the arterial iodide. In Experiment C-2, TSH given 45 minutes before perchlorate led to the release of stable and radioactive PBI and iodide, and perchlorate enhanced the discharge of iodide. The data (Table 4) indicated the much lower specific

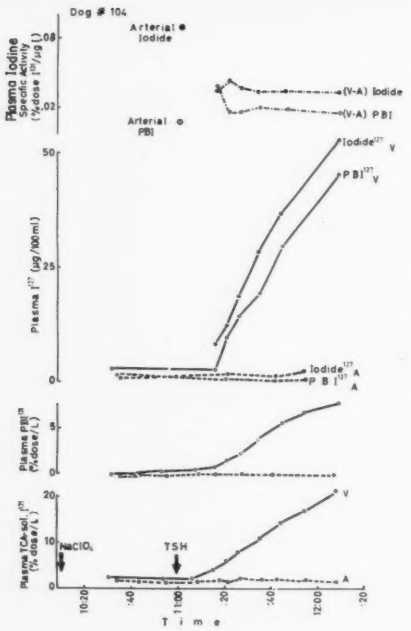

FIG. 5. Group C experiment. The animal was given I^{131} 48 hours before operation and perchlorate 20 minutes before thyroid venous cannulation. TSH led to the release of hormonal and acid-soluble iodine of reasonably constant specific activity.

activity of the iodide released from the gland as compared with systemic circulating iodide, and also showed this value to be more comparable to that of the glandular iodotyrosine-iodine than to that of the thyroxine iodine present in the gland and released into the circulation.

DISCUSSION

The results described support the idea previously proposed (1) that one of the prompt consequences of TSH administration is the appearance within and the release from the thyroid of iodide derived from an intraglandular source other than iodide newly "trapped" from the circulation.

TABLE 4. SPECIFIC ACTIVITY OF PLASMA AND THYROID IODINE, EXPERIMENT C-2

Iodine-containing material	Specific activity (% dose $I^{131}/\mu gI$)		
	Before TSH	45 minutes after TSH	After perchlorate
Arterial iodide	0.026	0.041	0.038
Thyroid venous iodide	0.024	0.015	0.014
Arterial PBI	0.0031	0.0022	0.0021
Iodide released from gland (V-A)	—	0.011	0.013
Glandular iodotyrosines*	—	—	0.0088
PBI released from gland (V-A)	—	0.0039	0.0039
Glandular thyroxine†	—	—	0.0034

* Butanol-soluble iodine re-extractable in Blau reagent.
† Butanol-soluble iodine not re-extractable in Blau reagent.

In addition, the data provide some suggestions as to the exchangeability, under different conditions, of plasma iodide entering the gland with the thyroidal iodide presumed to be formed within the thyroid.

The administration of TSH in experiments in which organic binding of iodide was permitted (group A experiments) led in most cases to a rise in the thyroid-venous concentration of stable iodide above the arterial value, indicating a net loss of iodide from the gland, with no consistent change in the thyroid clearance of plasma iodide. This indicated that iodide might be lost from the gland while accumulation of iodide from the plasma continued and suggested that the iodide accumulated by the gland from the plasma was not freely exchangeable with the iodide simultaneously generated within and released from the gland as a result of TSH stimulation, since one might expect that if exchange were appreciable, glandular release of stable iodide would have been associated with release of some I^{131} and consequent decrease in the extraction ratio. Although in some experiments decreases in the extraction ratio and clearance of plasma I^{131} were observed after TSH was given, similar observations were recorded in at least one control experiment, and this might have been caused by deterioration of the preparation or (as in experiments where the rate of decrease of the arterial plasma I^{131} concentration was unusually great) by incomplete equilibration of the tracer I^{131} with extrathyroidal iodide and consequent change in the specific activity of circulating iodide, and need not necessarily imply a decrease in thyroidal accumulation of plasma iodide induced by TSH.

Inactivation of the iodide-trapping mechanism by perchlorate approximately 1 hour after TSH administration in the group A experiments produced only a transient and relatively small release of I^{131} not appreciably greater than that observed after giving perchlorate to animals that had not received TSH; TCA-soluble I^{127} release, however, was sustained and appreciable, further suggesting the unlikelihood of appreciable admixture within the gland of labeled iodide entering from the circulation with iodide produced within the gland. This interpretation of the results is based on the following considerations. If there were considerable exchange between circulating iodide (labeled) "trapped" by the gland and intraglandular iodide not newly entered from the circulation, and if the quantity of iodide formed within the gland was comparatively large, so that the specific activity of the iodide mixture in the gland would be much lower than the specific activity of circulating iodide, then it is conceivable that iodide of low specific activity might leave the gland as a consequence of TSH injection without appreciably altering the thyroid extraction ratio for I^{131}. However, under these circumstances, although one might then have expected that the iodide released after perchlorate injection would have been of low specific activity, it would seem that the quantity of I^{131} dis-

charged from the gland after perchlorate might reasonably have been expected to be appreciably greater than was found.

The release of appreciable amounts of stable iodide from the gland following perchlorate injection in 3 of the 5 control experiments in group A associated with the simultaneous discharge of relatively small quantities of I^{131} suggests that even in the absence of exogenous TSH the gland may contain iodide, presumably derived from deiodination, which, during a short period of observation, is poorly exchangeable with iodide entering the gland from the circulation. It would appear that the thyroid/plasma concentration gradient for iodide from both sources is maintained by mechanisms subject to perchlorate inhibition.

The suggestion that the thyroid contains iodide not exchangeable with plasma iodide has been proposed as a possible explanation for the large discrepancy between the values for the thyroid iodide concentration determined chemically and the much lower values estimated from thyroid-serum iodide-I^{131} ratios (8).

The fact that there was no significant difference between the TSH-treated dogs and the controls in the average quantity of stable iodide discharged from the gland following perchlorate injection might at first suggest that the effect of TSH upon enhancing iodide release in the pre-perchlorate period was not reflected in changes in the poorly-exchangeable, perchlorate-dischargeable iodide stores within the gland. It might perhaps be emphasized, however, that under the experimental conditions, release of stable iodide was still taking place in both the TSH-treated dogs and in some controls at the end of the period of blood sampling after perchlorate injection, and the data cannot, therefore, be interpreted as indicating that TSH had no effect upon the quantity of stable iodide ultimately dischargeable from the gland by perchlorate. Indeed, in earlier studies (1) as well as in group A Experiments 15 and 16 and the group C experiments, a considerable increase in iodide release was observed following TSH administration to dogs pre-treated with perchlorate. On the other hand, under the present experimental conditions, the glandular release of radioiodide had been largely completed by the end of the period of observation following injection of perchlorate, and the absence of a significant difference in the quantity of I^{131} released between the control and TSH-treated dogs in group A would suggest that TSH had little effect upon the quantity of iodide accumulated by the gland from the circulation and existing as iodide within the thyroid.

The absence of an early (i.e. within 1–2 hours) effect of TSH upon glandular accumulation of circulating iodide recorded in these experiments in the dog, is consistent with the latent period of 8–10 hours noted by others between injection of TSH and enhancement of glandular accumulation rate in man (9, 10). A decrease in the rate of disappearance of iodide-I^{131} from the plasma has been reported to occur 12 to 24 hours after giving

TSH to human subjects under experimental conditions such that appreciable labeling of glandular organic iodine stores might have been achieved by the time this effect was apparent, and this effect has been attributed to the release from the gland of iodide derived from deiodination, to early inhibition of thyroid accumulation of circulating iodide, or both (11). The results of the present study in the dog, if applicable to man, suggest that the effect might be predominantly due to release of labeled iodide from the gland rather than to inhibition of glandular uptake of plasma iodide. It is of interest that in one reported series of experiments in man, apparent inhibition of radioactive iodine accumulation was observed 2 hours after TSH injection (12) but repetition of the study failed to reveal significant inhibition (or stimulation) of iodine uptake by TSH at this time interval (10, 12).

In the experiments of group B, in which organic binding had been prevented, the most striking effect of TSH was the almost immediate release of iodide-I^{131} from the gland, generally accompanied by release of appreciable quantities of stable iodide. This suggested that under these conditions, in contrast to the situation where organic binding was unimpeded, the iodide derived from deiodination reactions was at least to some degree exchangeable with iodide entering from the plasma. It would seem reasonable to attribute the TSH-induced discharge of trapped iodide-I^{131} to admixture of the increment of stable iodide produced within the gland (by deiodination of unlabeled organic iodine compounds) with labeled iodide in the thyroidal "iodide space," which would tend to increase the value of the thyroid/serum iodide ratio. As a result of the disturbed equilibrium, the rate of discharge of iodide from the gland might exceed the rate of entry from the circulation, with consequent release of labeled iodide from the gland. The release of trapped iodide-I^{131} under these circumstances, might be comparable, according to this view, to the "wash-out" of trapped I^{131} from the blocked gland observed when stable iodide is given (13), except that under the described experimental conditions the source of the stable iodide leading to the release of iodide from the gland would be not the plasma but the intraglandular precursors of iodide. Such a formulation could account for the fact that the specific activity of thyroid venous iodide often decreased after TSH, suggesting admixture with unlabeled iodide of the iodide trapped in the gland from the circulation, and it might account for the initial rise and subsequent fall of the venous iodide-I^{131} concentration occurring after TSH. If this explanation were correct, one might anticipate that TSH administered when the thyroid iodide was in equilibrium with circulating iodide might induce a greater release of trapped iodide-I^{131} from the gland when the thyroid/plasma iodide gradient was small than when it was large. Thus, if the same quantity of stable iodide were introduced into the thyroid iodide space when it contained a) a small or b) a large quantity of trapped labeled iodide of the same specific activity, one might

expect that restoration of the pre-existing equilibrium thyroid/plasma stable iodide ratio would entail the loss of a larger fraction of the glandular content of radioactive iodide in a) as compared with b). That this may indeed occur was suggested by the experiment shown in Figure 4, in which the thyroid/plasma iodide gradient had almost certainly been decreased by the small quantity of perchlorate given before TSH injection, and in which administration of TSH produced an unusually large release of iodide-I^{131}.

The possibility that the effect of TSH upon the release of labeled iodide from the blocked gland might be attributable to contamination of the TSH solution with thiocyanate, perchlorate or with iodide seems excluded by the finding that a boiled preparation of TSH did not produce this effect, and chemical analysis of the contents of a vial containing 10 units of TSH revealed only 0.015 μg of iodine

The specific activity of iodide initially released from the gland after perchlorate administration in the TSH-treated animals of group B-1 was much smaller than the corresponding values in the control group B-2. This also suggested that in the TSH group an appreciable fraction of the iodide in the "trap" one hour after TSH administration was not immediately derived from the plasma. By contrast the value of specific activity of the iodide released from the gland immediately after perchlorate injection in the control experiments was much closer to the specific activity of arterial iodide, suggesting that most of the iodide in the unstimulated gland had been accumulated from the circulation. It should perhaps be pointed out, however, that in 3 of the 4 control experiments, the specific activity of the iodide released after perchlorate injection was somewhat lower than the arterial values, suggesting again that even in the absence of exogenous TSH some of the glandular iodide may have been derived from sources other than the circulation. This inference is also supported by the finding that in the initial control period of the B-1 experiments, when iodide-I^{131} equilibrium seemed to exist between gland and plasma, the specific activity of the iodide in thyroid venous plasma tended to be less than that in arterial plasma.

The fact that the specific activity of the iodide released from the blocked gland after perchlorate became progressively smaller with time may be attributed to continuing deiodination of virtually unlabeled organic iodine compounds and release of the iodide so derived after labeled iodide which had been accumulated from the circulation had been largely discharged from the gland following inhibition of the trapping mechanism.

The results of the group C experiments, indicating that the specific activity of the iodide released from the gland as a consequence of TSH injection was much more like that of the glandular iodotyrosine iodine than of the thyroxine iodine, suggest the possibility that the released iodide may have originated from deiodination of the iodotyrosines. Although the iodide released from the thyroid was of somewhat higher specific activity

than the butanol-soluble iodine extracted by the Blau reagent, it should be noted that the "iodotyrosine" fraction separated in this way contains mono- as well as diiodotyrosine, and is not completely free of thyroxine (14), and since the specific activity of thyroxine (Experiment C-2) was low the specific activity of the iodotyrosine iodine could have been higher than the value found for the alkali soluble iodine. Further studies of the specific activity of the released iodide and of iodotyrosine iodine at different times after I^{131} administration and with resolution of mono- and diiodotyrosine might help to define the suspected precursor.

The view that TSH enhances the production of iodide within the thyroid and that when organic binding is blocked the iodide so formed is exchangeable with iodide trapped from the circulation is consistent with the observation that thyrotropin injection produced a prompt reduction in the concentration of iodide-I^{131} in the thyroid of man and of the rat when organic binding of iodine had been prevented by propylthiouracil or methimazole given a short time before the tracer I^{131} (15).

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THE EFFECT OF PROPYLTHIOURACIL, METHYLTHIOURACIL AND THIOURACIL ON THE PERIPHERAL METABOLISM OF *l*-THYROXINE IN THYROIDECTOMIZED, *l*-THYROXINE MAINTAINED RATS

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ABSTRACT

Thyroidectomized, *l*-thyroxine maintained rats were equilibrated isotopically with exogenous I^{131} labeled *l*-thyroxine and later treated with propylthiouracil, methylthiouracil or thiouracil. The ensuing alterations in the distribution of radioactive compounds were determined. The results obtained showed that these goitrogens depress extrathyroidal deiodination of *l*-thyroxine to about half its normal amount; subsequently a larger fraction of the hormone is excreted via the fecal route. Concentration of labeled compounds in the peripheral tissues is not greatly influenced, whereas the level of circulating hormone is usually increased above normal values. It is concluded that these "antithyroid" drugs have also a very rapid and intense action on the peripheral metabolism of *l*-thyroxine.

FREQUENT use has been made of the "antithyroid" properties of thiouracil and related compounds. It is well established that these drugs inhibit the biosynthesis of thyroid hormones by impairing the process of incorporation of iodine into organic compounds (1, 2). It has been widely assumed and more or less explicitly stated, that these goitrogens have no effect on the extrathyroidal metabolism of thyroid hormone(s), the main experimental basis for this statement being that the exogenous administration of thyroid hormone prevents or reverts alterations induced by them. Much of our present knowledge of thyroid-pituitary interrelationships and of thyroid hormone secretion and peripheral requirements obtained by goiter prevention techniques, for instance, proceed from experiments in which this assumption is implicit.

Andik *et al.* (3), however, showed that thyroidectomized rats treated with methylthiouracil require larger doses of thyroxine during longer periods for their lowered oxygen consumption to revert to normal than do untreated thyroidectomized animals. These findings indicated that the drug had produced peripheral alterations which interfered with the metabolic activity of thyroxine. Recently van Middlesworth (4) has seriously questioned the validity of goiter-prevention techniques for estimation of thyroid hormone secretion rates, having found that the doses of *l*-thyroxine

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which prevent appearance of goiter in propylthiouracil treated rats raise the level of circulating thyroid hormone well above normal. Another observation which is not in agreement with classical explanations of goitrogenesis induced by these drugs is that reported by Rawson (5) who observed that administration of propylthiouracil resulted in an altered thyroid histology as soon as 24 hours after onset of treatment, at which time the concentration of circulating thyroid hormone was still normal. These observations are suggestive of a peripheral alteration of thyroid hormone metabolism induced by these drugs.

To our knowledge, a satisfactory direct experimental approach to the problem has not been undertaken.[1] We therefore proposed to investigate whether thiouracil (TU),[2] methylthiouracil (MTU) and propylthiouracil (PTU) influence extrathyroidal metabolism of *l*-thyroxine, by using thyroidectomized, *l*-thyroxine maintained rats in isotopic equilibrium with exogenous I^{131} labeled *l*-thyroxine.

MATERIALS AND METHODS

General procedure:

Young male adult rats of a Wistar strain, weighing about 200–250 gm. at time of necropsy, were used for the present study. They were fed 15 gm./day/rat of a modified Remington low iodine medium residue diet at least 2 weeks prior to thyroidectomy until the end of the experiment. Calcium carbonate and vitamin D were added to the diet after thyroidectomy.

The animals were thyroidectomized surgically; in order to ensure completeness of removal of aberrant or residual thyroid tissue, most experimental groups received a tracer dose of radioiodide several days after operation. Measurements were taken over the neck, thymus area and thigh, animals whose data showed presence of thyroid tissue being discarded. The remaining animals received thereafter as daily intraperitoneal maintenance dose 4 μg *l*-thyroxine (*l*-T_4) in 0.5 ml. saline containing 2% rat serum. We had previously determined that thyroidectomized rats of this weight and strain fed the same diet required such a dose to maintain their plasma PBI within the same limits as non-operated animals under the same nutritional and environmental conditions. During this period the animals were trained to live in individual glass metabolism cages.

Determination of hormone concentrations; isotopic equilibrium:

The technique of isotopic equilibrium described by van Middlesworth (6) for intact rats was modified and extended to thyroidectomized, *l*-thyroxine maintained animals. About 2–3 weeks after thyroidectomy the maintenance dose was substituted by a daily intraperitoneal injection of 4 μg of I^{131} labeled *l*-thyroxine. When the individual urinary and fecal excretion curves had reached a plateau and approximately all of the injected

[1] After completion of the experimental work reported here, our attention was drawn to the fact that P. P. van Arsdel and R. A. Williams (11) had previously investigated the problem using quite different methods and experimental conditions, reaching results with which the present ones are in general agreement.

[2] In the present paper the following abbreviations are used: Thiouracil (TU); methylthiouracil (MTU); propylthiouracil (PTU); *l*-thyroxine (1-T_4); protein-bound iodine (PBI); thyroid-stimulating hormone (TSH); trichloroacetic acid precipitable (TCA pptbl.); 2,4-dinitrophenol (DNP).

dose was recovered daily in the excreta, the animals were considered in isotopic equilibrium with the exogenous labeled hormone. A few days later 15 mg. PTU (MTU or TU)/rat/day were added to the feed of half of the experimental animals during varying intervals.

The animals were then sacrificed and the I^{131} distribution determined throughout the body as described previously (7). The procedure for the determination of the trichloroacetic acid (TCA) precipitable fraction of I^{131} in plasma and carcass was modified, thiourea being added prior to fractionation, in order to avoid spurious data due to the possible presence of hemoglobin (8). If the radioactivity of a trachea suggested presence of thyroid tissue, the data of the corresponding animal were discarded.

The radioactivity of a given sample was expressed as percentage of the daily dose. Considering that the animals were in isotopic equilibrium with the labeled hormone till the end of the experiment and that the daily dose contained 4 μg *l*-T_4 (=2.7 μg I), the corresponding minimum iodine content of a sample may be calculated.

Determination of I^{131} labeled l-thyroxine disappearance rates:

Thyroidectomized rats, on 4 μg *l*-thyroxine maintenance dose, were injected into the femoral vein, under slight ether narcosis, with 4 μg I^{131} labeled *l*-thyroxine (1 μC) dissolved in 0.5 ml. of saline containing 2% rat serum. Immediately thereafter half the animals were started on 5 mg. of PTU every eight hours, the other half received an equal volume of saline, and were killed at different intervals after the injection of the labeled hormone and PTU.

I^{131} distribution was determined as described above, radioactivities being expressed as percentage of the injected dose. Obviously, under such experimental conditions concentrations of iodinated compounds may not be calculated from radioactivity data alone.

Excretion of I^{131} labeled iodide:

Thyroidectomized rats, in isotopic equilibrium with 4 μg I^{131} labeled *l*-thyroxine, were treated with PTU as described above. On the 7th day after the onset of treatment with the goitrogen, these animals and their controls received an intraperitoneal injection of 2.7 μg of I^{131} labeled iodide of the same specific activity as that corresponding to the hormonal maintenance dose on the same day.[3]

The excreta were collected for a few days more, the daily injections of labeled hormone being continued throughout the experiment.

Preparation of I^{131} labeled l-thyroxine solutions:

I^{131} *l*-thyroxine, labeled in the 3′:5′ position, was either obtained from Abbott Pacific Ltd., or synthesized by us from *l*-diiodothyronine and purified on an anion exchange resin (Dowex, 1 X-10, 200–400 mesh). The specific activity was determined in the purified eluate by modification of a technique previously reported (9). Stable *l*-thyroxine was added to give a final solution with a total of 4 μg of *l*-thyroxine in 0.5 ml. of saline containing 2% rat serum. The specific activity of the solution differed for the various experimental groups, being about 0.4–0.8 μc/4 μg of *l*-T_4 (as determined on the day of the first injection).

RESULTS

Figure 1 shows typical fecal and urinary excretion curves obtained for one of nine experimental groups of thyroidectomized *l*-thyroxine main-

[3] This procedure was considered the most suitable in order to avoid the possible differences in specific activity of the excreted iodide, which might arise between control and PTU treated animals, if the goitrogen influenced the deiodination of *l*-thyroxine.

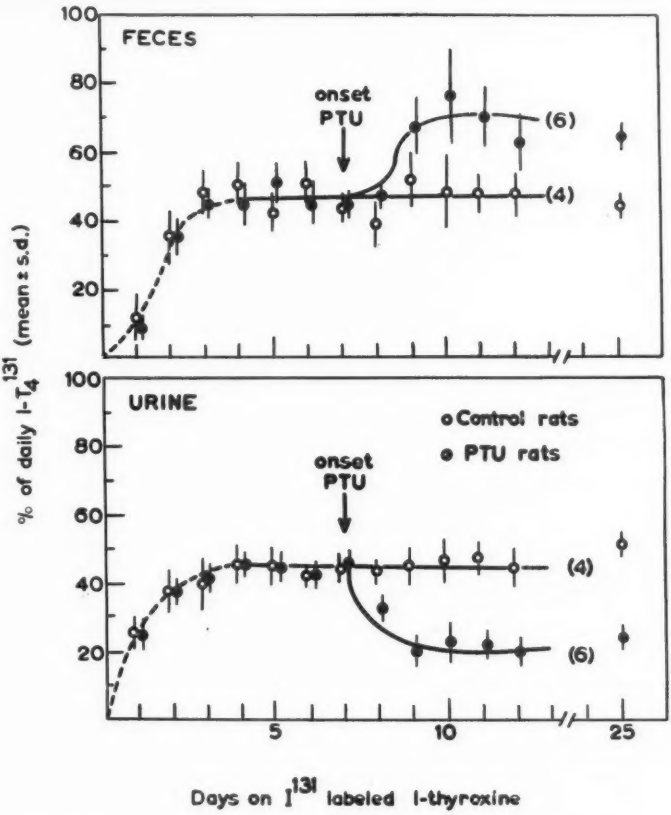

FIG. 1. The influence of PTU (15 mg./rat/day) on the urinary and fecal excretion of iodinated compounds in thyroidectomized rats, isotopically equilibrated with 4 μg I^{131} labeled *l*-thyroxine/rat/day. Data are expressed as percentage of the daily injected dose, the mean ± s.d. being shown; the number of animals of the group is shown in the brackets.

tained rats in isotopic equilibrium, before and during the addition of 15 mg. PTU/day/rat to the feed.

As may be observed from these results, the animals were isotopically equilibrated with the I^{131} labeled *l*-thyroxine after 4–5 days and remained so till the end of the experiment. The urinary radioactivity (chromatographically over 97% iodide) decreased during the first 24 hours of PTU treatment, reaching about half the control value (mean $P < .001$) by the second day and remaining so during administration of the drug. The fecal excretion of radioactive compounds started increasing about 2 days after the onset of goitrogen treatment, reaching a level which compensated for the decreased urinary output. Later experiments showed that both urinary

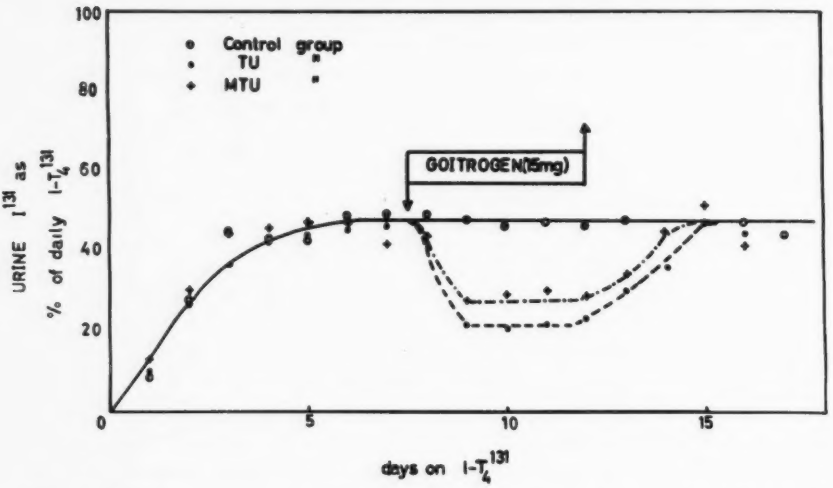

FIG. 2. The urinary excretion of iodide in thyroidectomized, thyroxine maintained, isotopically equilibrated rats, before and during treatment with methylthiouracil (MTU) and thiouracil (TU) and after withdrawal of the drug.

and fecal excretion of iodinated compounds returned to control values in about two days if the drug was discontinued.

The excretion curves of animals treated with 15 mg./rat/day of MTU or TU showed qualitatively the same variations as those described for PTU treated rats, as may be seen in Figure 2.

Tables 1 and 2 show the I^{131} distribution for two different experimental groups. As may be observed, the increased I^{131} content of the intestinal tract correlates well with the increased fecal excretion of labeled compounds mentioned above. The I^{131} content of stomach (mostly iodide) was decreased, and this finding is in agreement with the decreased urinary excre-

TABLE 1. THE EFFECT OF PROPYLTHIOURACIL ON THE I^{131} DISTRIBUTION IN ISOTOPICALLY EQUILIBRATED THYROIDECTOMIZED RATS*

	PTU during 5 days % daily dose: mean ± s.d.		Statistics: P	PTU during 18 days % daily dose: mean ± s.d.		Statistics: P
	PTU treated (6 rats)	Control (4 rats)		PTU treated (3 rats)	Control (4 rats)	
1 ml. Plasma	1.82 ± .22	1.65 ± .23		1.69 ± .13	1.63 ± .28	
TCA pptbl. in 1 ml. Plasma	1.76 ± .21	1.65 ± .25		1.63 ± .05	1.50 ± .24	
1 gm. Carcass	.20 ± .02	.18 ± .02		.20 ± .01	.20 ± .02	
TCA pptbl. in 1 gm. Carcass	.18 ± .02	.17 ± .02		.16 ± .01	.16 ± .02	
Liver	13.7 ± 1.8	12.3 ± 1.3		15.9 ± 4.1	11.6 ± .5	
Intestines (+contents)	38.6 ± 5.6	26.9 ± 4.7	.01	41.0 ± 6.1	29.9 ± 4.3	.05
Feces	63.5 ± 11.3	47.9 ± 5.5	.02	63.3 ± 3.9	43.1 ± 3.2	.001
Urine	21.0 ± 1.9	46.4 ± 4.9	$<.001$	24.9 ± 1.8	50.3 ± 2.2	$\ll.001$
Stomach	1.69 ± .83	3.42 ± .27	$<.001$	1.97 ± .32	4.30 ± .93	$<.02$

* Daily maintenance dose: 4 μg I^{131} labeled *l*-thyroxine.
PTU dose: 15 mg./rat/day.
Animals were killed 18 hours after last labeled hormone injection.

TABLE 2. THE EFFECT OF PROPYLTHIOURACIL ON THE I^{131} DISTRIBUTION IN ISOTOPICALLY EQUILIBRATED THYROIDECTOMIZED RATS*

	PTU treated 8 rats	Control 6 rats	Statistics: P
	% daily dose: mean ± s.d.		
1 ml. Plasma	1.38 ± .14	1.06 ± .16	.001
TCA pptbl. in 1 ml. Plasma	1.21 ± .11	.91 ± .11	< .001
1 gm. Carcass	.46 ± .06	.48 ± .07	
TCA pptbl. in 1 gm. Carcass	.44 ± .07	.46 ± .08	
Liver	31.1 ± 5.1	31.7 ± 5.9	
Intestines (+contents)	46.2 ± 10.0	28.2 ± 7.2	.01–.001
Intestines + Feces	113.5 ± 16.1	88.0 ± 8.8	.01–.001
Urine	16.1 ± 1.8	29.5 ± 3.3	≪.001
	Caracass/Plasma ratio		
Total I^{131}	.33 ± .04	.43 ± .06	.01–.001
TCA pptbl. I^{131}	.36 ± .04	.53 ± .05	< .001

* Daily maintenance dose: 4 μg I^{131} labeled *l*-thyroxine.
PTU dose: 15 mg./rat/day during 6 days.
Time of necropsy: 24 hours after last labeled hormone injection.

tion. The mean concentration of total iodinated compounds in the plasma and the corresponding TCA precipitable fraction were the same or higher for PTU treated animals as compared to the mean control values, the differences being statistically significant for most of the experimental groups (in 5 out of 7, with a 31% mean increase). The concentration of labeled compounds in the carcass[4] was the same as that of control animals or was decreased. This was reflected in the ratio between carcass and plasma total or TCA precipitable radioactivity which was usually lower for the animals under PTU treatment. The radioactivity of the heart, kidneys and spleen was the same or higher for PTU treated animals as compared to the controls.

These results suggested that the overall peripheral deiodination of *l*-thyroxine decreases intensely and rapidly after the administration of either PTU, MTU or TU. However, although unlikely, the possibility existed that deiodination was not impaired, the decreased urinary iodide reflecting some kidney damage, with part of the iodide being then deflected to excretion via the feces. Reliable quantitative chromatographic analysis of the latter was not feasible due to the very low radioactivities of the samples and this point was therefore investigated by injecting thyroidectomized rats, in isotopic equilibrium with exogenous labeled thyroxine and treated with PTU, with labeled iodide as described above. Figure 3 shows that all the injected iodide was excreted into the urine in the same manner both by treated and control animals, no increase in radioactivity due to the I^{131} iodide being detectable in the feces.

[4] By carcass the mechanically homogenized skeletal muscle + skeleton is understood.

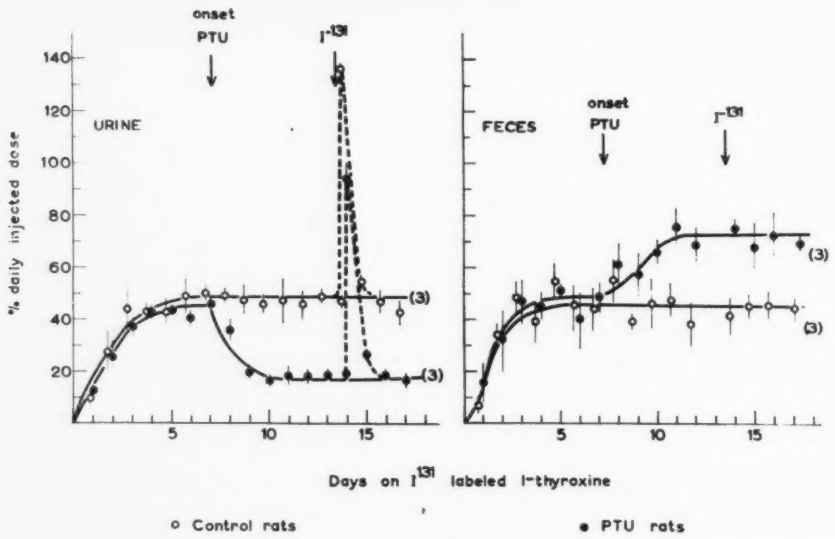

FIG. 3. Urinary and fecal excretion of labeled iodinated compounds before and after the injection of a single dose of labeled iodide (2.7 μg) into thyroidectomized rats, previously isotopically equilibrated by daily injection of 4 μg labeled *l*-thyroxine, with and without PTU treatment (15 mg./rat/day).

We also believed it interesting to elucidate whether the urinary iodide excretion decreases as a primary effect of thiouracil administration, the increased fecal excretion being secondary to it, or whether the latter was the primary effect and the decreased urinary iodide merely reflected that less hormone was available to the peripheral tissues for deiodination. This was investigated by measuring I^{131} labeled *l*-thyroxine disappearance as described above. Table 3 shows that as early as 5 hours after the onset of PTU administration a 30% decrease of urinary iodide I^{131} was detectable, whereas there was no increase of the hepatic, intestinal or fecal radio-

TABLE 3. THE EFFECT OF PROPYLTHIOURACIL ON THE I^{131} DISTRIBUTION IN THYROIDECTOMIZED *l*-THYROXINE MAINTAINED RATS AT DIFFERENT INTERVALS AFTER THE INJECTION OF 4 μG I^{131} LABELED *l*-THYROXINE*

	5 hours after I^{131} *l*-T_4 % daily dose: mean ± s.d.			20 hours after I^{131} *l*-T_4 % daily dose: mean ± s.d.		
	PTU treated	Controls	Statistics: P	PTU treated	Controls	Statistics: P
TCA pptbl. in 1 ml. Plasma	1.50 ± .14	1.35 ± .16		.77 ± .08	.63 ± .08	.05–.02
TCA pptbl. in gm. Carcass	.14 ± .01	.13 ± .01		.078 ± .007	.079 ± .004	
Liver	14.9 ± 1.6	13.8 ± .7		11.2 ± .4	10.7 ± .9	
Intestines (+contents) + Feces	24.2 ± .5	25.6 ± 2.1		49.8 ± 5.1	41.6 ± 1.8	<.01
Urine	3.1 ± .6	4.8 ± .3	.01–.001	5.3 ± 2.2	15.0 ± .9	≪.001
Stomach				.44 ± .13	1.08 ± .24	<.001
	Carcass/Plasma Ratio			Carcass/Plasma Ratio		
Total I^{131}	.10 ± .02	.10 ± .03		.10 ± .01	.13 ± .01	.001–.001
TCA pptbl. I^{131}	.09 ± .01	.10 ± .02		.10 ± .01	.13 ± .01	.05

* PTU dose: 5 mg./rat/8 hrs. starting immediately after the injection of labeled hormone.

activity. By 20 hours after onset of PTU administration there was a 60% decrease of the urinary radioiodide, a 20% increase of the intestinal+fecal radioactivity being by then demonstrable, together with the other typical alterations of the peripheral I^{131} distribution pattern. Moreover, in a few thyroidectomized, isotopically equilibrated rats, bile was collected from the common duct during the first hours after onset of PTU administration and it was then found that during the first 7 hours the urinary radioactivity had decreased significantly, the mean being 3.8% of the daily dose for the PTU treated animals versus 10.6% for the controls. The radioactivity of the bile collected over this period was quite the same for both groups of rats (20.9% and 21.5% respectively). When bile was collected from animals which had been 4 days on PTU, the urinary iodide was sharply decreased, as usual, and by then an increase in the biliary radioactivity was observed. It appears, therefore, that a decrease in the overall peripheral deiodination of *l*-thyroxine is the first effect of PTU administration clearly detectable in this type of experiment and that the delay in the increase of the fecal excretion is not only due to the time necessary for the formation of feces. The present experiments do not, however, permit an elucidation of whether the increased fecal excretion of iodinated compounds is an independent effect of PTU administration or is due to thyroxine accumulating in the tissues because of impaired deiodination, nor whether it is due to increased biliary secretion alone or is also enhanced by an impaired intestinal reabsorption.

DISCUSSION

Kidney impairment of renal iodide excretion has been excluded experimentally in the present paper as a possible cause for the decreased urinary iodide observed for thyroidectomized, *l*-thyroxine maintained rats during treatment with PTU, MTU and TU. A decreased availability of the hormone to the peripheral tissues was also excluded as a possible cause: it has been shown here that a decrease in urinary I^{131} is already clearly demonstrable at a moment when biliary secretion and/or the intestinal+fecal content of iodinated compounds is not yet altered. Neither in such experimental groups nor in those where both decreased urinary iodide and increased fecal excretion of iodinated compounds were observed, was the concentration of such compounds in peripheral tissue significantly lower for PTU treated than for untreated controls.

It was therefore concluded that propylthiouracil, methylthiouracil and thiouracil at the dose level here employed (.1% of the feed) has a rapid and intense effect on the extrathyroidal metabolism of *l*-thyroxine in the rat: of the different changes observed the quantitatively most striking is that the deiodination of *l*-thyroxine, at least in the 3′,5′ positions, is decreased to half or less the normal amount.

As we have previously referred to at the beginning of the present paper,

Andik *et al.* (3) found that to restore to normal the lowered oxygen consumption of thyroidectomized rats, thyroxine requirements increased if the animals were concomitantly treated with methylthiouracil. Very recently Stasilli *et al.* (10) have also reported that the calorigenic effectiveness of thyroxine is greatly decreased by thiouracil feeding. Our findings are not contradictory, since the possibility exists that there is some more or less direct relationship between the metabolic effectiveness of the thyroid hormone and its catabolism in the tissues via deshalogenating pathways. By this statement it is not meant that the relationship, if any, is necessarily of a causal nature: variations of the peripheral deiodination of the hormone and of its metabolic effectiveness may be different indexes of the disturbance of other metabolic parameters.

Van Middlesworth *et al.* (4) have seriously questioned the validity of goiter-prevention techniques for the evaluation of thyroid hormone secretion rates, stating the possibility that values thus obtained are erroneously high. Our present results support this point of view, since such tests are implicitly based on the assumption that the goitrogen does not have a direct effect on the peripheral metabolism and effectiveness of the thyroid hormone. However, it appears that a same dose of thyroxine in a PTU-treated and in an untreated animal are not equivalent: from the present data it seems valid to state that a smaller fraction of the injected hormone is metabolized via deiodination, a larger proportion is excreted undegraded via fecal route; as shown by others (3, 10) its metabolic effectiveness is likewise impaired.

We believe that the hypothesis might be taken into consideration that the alterations caused by PTU (MTU, TU) in intracellular metabolism,[5] alterations which are reflected in our data by a decreased deiodination of the hormone and in those of others (3, 10) by a decreased metabolic rate, might be influencing the secretion of TSH by the pituitary. It would then result that prevention of goitrogenesis would require larger doses than those equivalent to the secretion rate of a normal thyroid gland.

In order to test the validity or not of the assumptions stated above, experiments are now under way in our laboratory which have been planned reasoning along such lines.

As a final remark we wish to emphasize that in experiments planned to investigate peripheral aspects of thyroid hormone metabolism—such as secretion rates, peripheral requirements, activity of analogues, mode of action at the cellular level, etc.—animals treated with PTU (MTU, TU) ought to be avoided or at least not considered equivalent to thyroidectomized ones.

[5] Alterations not mediated via the direct effect of these compounds on the thyroid are referred to here.

Acknowledgments

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THYROXINE UPTAKE BY LIVER MITOCHONDRIA OF THE COLD-EXPOSED RAT

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ABSTRACT

Mitochondrial binding of thyroxine as a function of thyroxine concentration (1.0×10^{-5}M to 6.0×10^{-5}M) was studied in the livers of normal control, hyperthyroid and hypothyroid rats with and without cold exposure at 2–4° C. Binding studies were carried out using non-labeled L-thyroxine and measuring the ultra-violet absorbancy of thyroxine left after the separation of mitochondria from the incubating medium. In mitochondria from the normal rat, a linear relationship between the amount of thyroxine bound and the concentration in the medium was observed. In mitochondria of rats cold-exposed for 5 hours, there appeared to be a smaller rate of increase in thyroxine binding at the higher concentrations. In the case of rats refrigerated for 8 hours, the uptake of thyroxine was maximal at 3×10^{-5}M. The same linear relationship as for normal controls was found with mitochondria from hyperthyroid rats and 5 hours of cold-exposure did not exert any influence on this relationship. In mitochondria from hypothyroid rats, the relationship between thyroxine concentration and mitochondrial binding was similar to that for normal, cold-exposed animals.

The reduced rate of increase of bound thyroxine with increasing thyroxine concentration in mitochondria of both refrigerated and hypothyroid rats is interpreted as indicating a decrease in the number of available binding sites through their partial pre-saturation with thyroid hormone *in vivo*.

MANY experimental results support the "feed-back" hypothesis that at low environmental temperature, an increased rate of peripheral utilization, resulting in lowered blood level of hormone, may augment pituitary thyrotrophin (TSH) release to produce increased thyroid activity (1). Whereas oxygen consumption of animals is increased immediately after cold-exposure (2, 3), the increase of thyroid activity is not so definite in a short period of cold-exposure (4, 5, 6). An increase in peripheral utilization and in requirement of thyroxine at low environmental temperature has been demonstrated (2, 7–13). However, an increase in thyroid hormone uptake by peripheral tissues in which oxidative metabolism takes place has never been observed directly under the cold-exposed condition. Also assuming that the increase in metabolic rate in refrigerated animals does not depend on a hyperthyroid state but does depend on the presence of thyroid hormone (2, 11), there still remains a question; namely, does

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thyroid hormone play a significant role in the onset of the response to the cold stimulus?

Recently it has been demonstrated that thyroxine is taken up by rat-liver mitochondria *in vitro* (14, 15). The situation *in vivo* is so complex that no *in vitro* system at the subcellular level could be expected to achieve comparable conditions (15). It may be possible, however, to estimate the *in vivo* effect of cold on alteration in the mitochondrial binding sites from studies on the behavior of binding sites *in vitro*.

This paper is a report of studies carried out to examine the *in vitro* uptake of thyroid hormone by liver mitochondria of cold exposed rats.

MATERIALS AND METHODS

Male rats of the Wistar strain, weighing 130–170 gm., supplied from the laboratory of Dr. Makino, were used in these studies. They were housed at a room temperature of 24–27° C. Mitochondrial binding of thyroxine as a function of thyroxine concentration was studied using livers of normal, hyperthyroid and hypothyroid rats with and without cold-exposure. The hyperthyroid state was induced by intraperitoneally injecting L-thyroxine, 50 μg per 100 gm. body weight, for 5 consecutive days. They hypothyroid rats received daily injections of methylthiouracil, 4 mg. per 100 gm. body weight for 6 days. On the day after the final injection, the animals were sacrified for isolation of mitochondria. Cold-exposure was achieved by placing rats in a refrigerator at 2–4° C., for 2, 5, or 8 hours, with feed and water supplied *ad libitum*. Cold-exposed rats were sacrificed immediately after being removed from the refrigerator.

The binding studies described in the present paper were carried out by using non-labeled L-thyroxine-Na and measuring the concentration of thyroxine left in the supernatant after the separation of mitochondria from the incubation medium, whereas Klemperer (14) and Tapley and Basso (15) directly determined the amount of thyroxine bound to mitochondria. Mitochondria were isolated by suspending in a medium containing 0.25 M sucrose and 0.02 M tris-(hydroxymethyl)-aminomethane, pH 7.4, by the method of Schneider (16) with washing four times. The final pellet was suspended in 0.25M sucrose and 0.01 M phosphate buffer, pH 7.4, so that the mitochondria from 1 gm. of fresh liver were suspended in 1.0 cc. Fresh mitochondrial suspensions were used in all experiments.

To determine binding, the following three incubation systems, in plastic centrifuge tubes, were prepared for each concentration of thyroxine: The first tube contained 0.2 cc. of mitochondrial suspension, 4.3 cc. of 0.25M sucrose solution in 0.01M phosphate buffer (pH 7.4), and 0.5 cc. of L-thyroxine-Na (British Drug Houses Ltd.) in 0.005 M NaOH. Thyroxine was prepared just prior to use. The second tube contained the same amounts of mitochondria and other constituents without added thyroxine. The third tube contained the same amounts of medium as the first tube, but no mitochondria. In the first tube the binding reaction took place, and the second and third ones were prepared to allow correction for the non-precipitate of mitochondrial suspension and for a check of total thyroxine concentration in the incubation medium.

Following addition of the mitochondria, the tubes were inverted several times to ensure an even dispersion of the mitochondria, and then allowed to stand at 0–5° for 15 min. The tubes were then centrifuged at 13,000×g for 10 min. in a refrigerated centrifuge. 1-cc. aliquots of supernatant were diluted 10-fold with deionized water and their ultra-violet absorbancy was measured at a wavelength of 228 mμ. The ultraviolet absorption curve of L-thyroxine showed a maximum at 228 mμ in the medium containing 22.5 mM sucrose, 0.45 mM NaOH and 0.9 mM phosphate (pH 7.4), which

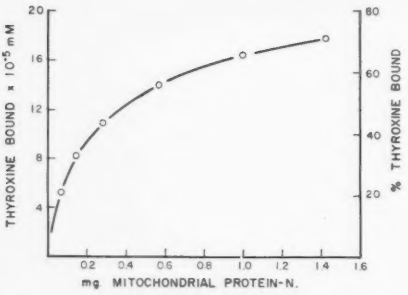

FIG. 1. Binding of L-thyroxine to rat-liver mitochondria as a function of the concentration of mitochondria. Thyroxine concentration; 5.0×10^{-5}M. Mitochondria: 0.05 cc., 0.1 cc., 0.2 cc., 0.4 cc., 0.7 cc. and 1.0 cc.

were the final concentrations after dilution. The concentration-optical density curve is linear in a thyroxine concentration range of 10^{-6} to 6×10^{-6}M. The dilution made the ultraviolet absorbancy of the non-precipitate of mitochondrial suspension very small (below 0.1 in optical density) and thus enabled measurement of the thyroxine concentration independent of the non-precipitate of mitochondria. The concentration of thyroxine left in the supernatant was obtained by subtracting the reading of the tube containing only mitochondria from the reading of the tube containing mitochondria+thyroxine. From the value thus obtained and the amount of total thyroxine added, the amount of thyroxine precipitated in contact with mitochondria was calculated. A single preparation of mitochondria obtained from the liver of an individual rat was used for each experiment. All results in this paper were confirmed by repeating each experiment at least four times. Results were expressed as mM bound or mM bound per mg. of protein-N of the mitochondria.

RESULTS

Uptake of thyroxine by mitochondria from normal rats

Figure 1 shows the effect of changing the numbr of mitochondria in the incubation medium. The amount of thyroxine bound was increased gradually but significantly with the increase in the number of mitochondria over the entire range studied.

The binding of thyroxine to mitochondria at concentrations of thyroxine ranging from 2.0 to 6.0×10^{-5}M is illustrated in Figure 2. A linear relationship between the amount of thyroxine bound and the concentration in the medium was observed over the whole range of thyroxine concentrations. It was interesting that the experiment was repeated six times and almost the same slope obtained each time.

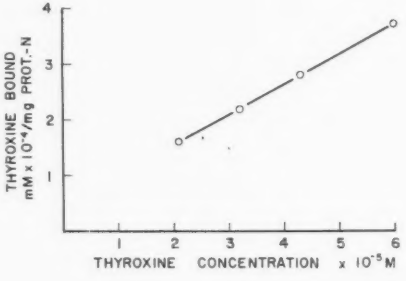

FIG. 2. Binding of L-thyroxine to liver mitochondria from normal rats as a function of thyroxine concentration (2.0×10^{-5}M to 6.0×10^{-5}M). Medium and incubation procedure as defined in the text.

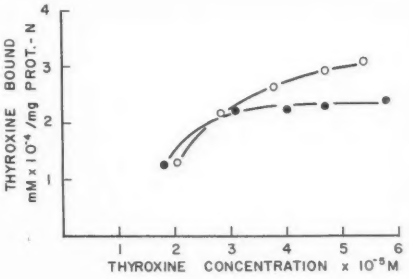

FIG. 3. Binding of L-thyroxine to liver mitochondria from cold-exposed rats as a function of thyroxine concentration (2.0×10^{-5}M to 6.0×10^{-5}M). Medium and incubating procedure as defined in the text. Open circle, cold-exposure for 5 hours; closed circle, cold-exposure for 8 hours.

Influence of cold exposure on the mitochondrial uptake of thyroxine as a function of thyroxine concentration

With mitochondria from rats refrigerated for 2 hours, degree of thyroxine binding as a function of thyroxine concentration increased linearly, as in the case of normal controls. But, by prolongation of cold-exposure to 5 hours, there appeared a decline in the rate of increase of bound thyroxine at the higher concentrations (Fig. 3). This was also apparent with mitochondria from rats cold-exposed for 8 hours (Fig. 3). In this case, the uptake of thyroxine was maximal at 3×10^{-5}M, above which the increase in concentration did not significantly increase the amount of thyroxine bound. These results indicacted. that the number of available binding sites in mitochondria isolated from refrigerated rats was smaller than in those from normal controls. It was supposed that this decrease in the number of available binding sites in refrigerated rats was due to partial *in vivo* pre-saturation of the binding sites by thyroxine or other competitive substances. As noted in the introduction, the first problem was whether or not this pre-saturation of mitochondrial binding sites was dependent on an elevated thyroid hormone level in the circulation. Therefore, this question was investigated in hyper- and hypothyroid rats with and without cold-exposure for 5 hours.

Binding of thyroxine as a function of thyroxine concentration in mitochondria from the hyperthyroid rat

If the decrease in number of available binding sites observed in mitochondria from normal refrigerated rats was due to a hyperthyroid state, then the mitochondria isolated from thyroxine-injected rats should behave as those from refrigerated rats. As illustrated in Figure 4, this was not the case. A linear relationship was observed between the amount of bound thyroxine and thyroxine concentration, indicating that the number of available binding sites was not different in hyperthyroid than in normal control rats in so far as could be determined with the concentrations used. Another interesting result was that 5 hours of cold-exposure did not exert an appreciable influence on the binding ability of mitochondria from the hyperthyroid rats (Fig. 4).

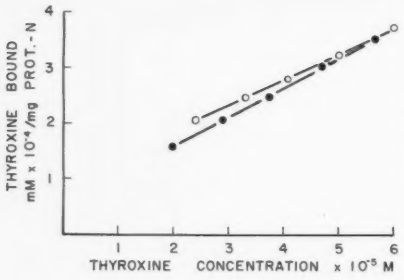

FIG. 4. Binding of L-thyroxine to liver mitochondria from hyperthyroid rats as a function of thyroxine concentration. Open circle, hyperthyroid control; closed circle, hyperthyroid rats cold-exposed for 5 hours.

Binding of thyroxine as a function of thyroxine concentration in the hypothyroid rat

Contrary to the results obtained using hyperthyroid animals, the mitochondria isolated from hypothyroid rats showed a lower rate of thyroxine uptake than the control at the higher concentrations (Fig. 5). This result indicated that the methylthiouracil treatment resulted in a decrease in the number of available binding sites, presumably through presaturation *in vivo*. It must be noted here again that 5 hours of cold-exposure did not cause a further alteration in the behavior of binding sites in mitochondria from hypothyroid rats (Fig. 5).

DISCUSSION

According to Tapley and Basso (15), the relationship between thyroxine concentration and the amount of thyroxine bound by rat mitochondria was linear at low thyroxine concentrations (less than 10^{-6}M); however, with higher concentrations of thyroxine (10^{-5} M and higher) the rate of increase of bound thyroxine lessened with increasing thyroxine concentration. This was interpreted as the result of a progressive saturation of the available binding sites. In the present work with normal rats, in contrast, a linear relationship was observed between the amount of bound thyroxine and the thyroxine concentration, using a range of thyroxine concentrations in which Tapley and Basso (15) found progressive saturation. Since their interpretation is plausible, mitochondria which exhibit saturation of

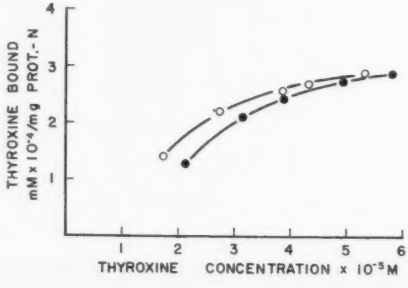

FIG. 5. Binding of L-thyroxine to liver mitochondria from hypothyroid rats a function of thyroxine concentration. Open circle, hypothyroid control; closed circle, hypothyroid rats cold-exposed for 5 hours.

thyroxine binding must have a smaller number of available binding sites than mitochondria which show a linear increase in thyroxine binding under the same experimental condition.

The experimental procedure in the present work is quite different from that of Tapley and Basso. The discrepancy between the present results and theirs may have resulted from either or both of the following differences:

First, with the method of Tapley and Basso, it is inevitable that loosely bound thyroxine is removed with washing. With the method reported here however, it is impossible to separate loosely-bound thyroxine from that which is firmly bound.

Second, differences in season or other environmental conditions, or genetic differences in rats may have existed.

Evidence has been presented in this paper that the number of available binding sites of liver mitochondria is decreased if the normal, intact animal is exposed to cold for several hours. These data on thyroxine binding by liver mitochondria resemble those obtained by Tapley and Basso in *normal* (not cold-exposed) rats, using the same range of thyroxine concentration. The reason for this discrepancy is not known.

In the light of previous investigations of reduced circulating thyroid hormone in cold-exposed animals (2, 7–13), the pre-saturation of binding sites in refrigerated rats is considered to be due to the increased *in vivo* uptake of endogenous thyroxine or other thyroid hormones by the mitochondria. And it was demonstrated in the present work that such an increased thyroxine uptake in refrigerated rats is not dependent on the hyperthyroid state, thus supporting the theory that increased peripheral utilization of thyroid hormone precedes increased secretion.

However, an unexpected finding in this study—namely, that the decrease in the number of available binding sites of mitochondria from hypothyroid rats was similar to that in cold-exposed rats—indicates that a complicated physiological mechanism is involved in uptake of thyroid hormone by liver mitochondria *in vivo*. Full elucidation of this mechanism is impossible at present, but the increased thyroxine uptake of mitochondria in the liver of both refrigerated and hypothyroid rats may be considered an adaptive response for retaining the available thyroid hormone in the peripheral tissues under conditions that require an increase in or preservation of metabolic rate. The evidence that, in hyperthyroid rats, cold-exposure did not exert further influence on the number of available binding sites, suggests that the adaptive response mentioned above is not operative when there is a high level of circulating thyroid hormones.

Better understanding of these phenomena remains to be gained in future studies.

Acknowledgment

The authors wish to express their gratitude to Dr. William J. Mellen for his kindly advice in the preparation of this manscript.

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THE EFFECT OF 1-(*p*-2-DIETHYLAMINOETHOXYPHENYL)-1-PHENYL-2-*p*-ANISYL ETHANOL UPON RAT PITUITARY AND UTERINE RESPONSES TO ESTROGEN[1]

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ABSTRACT

The pituitary hypertrophy which was readily induced in rats by chronic administration of estradiol valerate was inhibited by the concomitant administration of 1-(*p*-2-diethylaminoethoxyphenyl)-1-phenyl-2-*p*-anisyl ethanol (MER-25), an analogue of chlorotrianisene. Microscopic examination of the pituitary glands of the animals receiving estrogens showed a chromophobe hyperplasia with no evidence of an associated mammary hypertrophy. The combined administration of MER-25 and estradiol in doses which individually induced 50% gonadotropin inhibition in parabiotic rats resulted in a failure of MER-25 to block the pituitary inhibiting effect of estradiol. The pituitary glands of both the single and parabiotic rats which received only estrogen were found, upon bioassay, to be extraordinarily rich in gonadotropin content. This indicated that estrogen blocks the release rather than the production of gonadotropins. *In vitro* studies utilizing uterine strips reveal that specimens obtained from spayed rats treated with estradiol and MER-25 showed a decrease in amplitude of contraction as compared to tissues obtained from animals receiving estradiol alone. Interestingly enough, the uterine weights of animals receiving combined injections of MER-25 and estradiol valerate weighed less than the uteri of animals receiving estradiol alone. It may be concluded from these data that MER-25 is an anti-estrogenic compound in some respects but not for all modalities of estrogenic activity.

1-(*p*-2-diethylaminoethoxyphenyl)-1-phenyl-2-*p*-anisyl ethanol (MER-25), an analogue of the synthetic estrogen tri-*p*-anisyl chloroethylene (TACE) (Fig. 1), has been reported to antagonize the effects of both endogenous and exogenous estrogens. Various studies with rats have demonstrated that MER-25 prevents estrogen-induced pituitary hypertrophy, prevents castration hypertrophy of the pituitary, prevents estrogen-induced morphologic changes in the pituitary, does not alter pituitary gonadotropin content, and produces gonadotropin inhibition. MER-25 has also been shown to inhibit estrogen-induced uterotropic effects and vaginal

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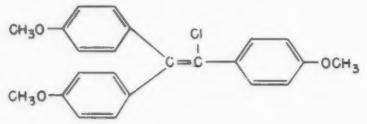
cornification as well as normal zygotic development (5, 15, 16, 23). Moreover, neither androgenic nor progestational activities have been demonstrated with this compound (9, 16). The studies reported herein were undertaken to confirm and extend the works cited in the hope that further investigation of the potentialities of this estrogen antagonist might prove useful in elucidating the mechanisms of action of estrogens. Observations were made on the effects of MER-25 upon three well-documented modalities of estrogenic activity: the induction of pituitary hypertrophy in rats by chronic estrogen administration, the inhibition of gonadotropins by estrogen as demonstrated by the parabiotic rat technique, and the estrogen enhancement of rat uterine contractions *in vitro*.

TACE

TRI-p-ANISYL CHLOROETHYLENE



MER-25

1-(p-2-DIETHYLAMINOETHOXYPHENYL)-1-PHENYL-2-p-ANISYL ETHANOL

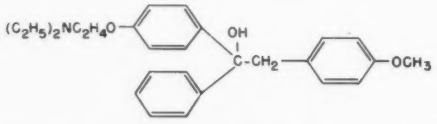


FIG. 1. Structural formulae of TACE and MER-25.

MATERIALS AND METHODS

Pituitary Hypertrophy in Intact Adult Female Rats

The effect of MER-25 upon the pituitary hypertrophy induced by chronic estrogen administration was evaluated by using four experimental groups of intact young adult 100 gram female albino rats. The first group remained untreated; 0.2 mg. estradiol valerate per week was administered to group two; group three was injected daily with 5.0 mg. MER-25;[2] and the fourth group was given combined estradiol and MER-25 therapy in the same respective doses. All injections were administered subcutaneously in volumes of 0.2 cc. per dose. Autopsies for organ weights were performed at monthly intervals from months three to eight of treatment. Representative pituitary glands were fixed in 10% formalin and histological sections were stained with Masson's trichrome and hematoxylin and eosin.

Since even relatively short term estrogen administration can elicit hypertrophic changes in the rat's pituitary and a qualitative rather than precise quantitative evaluation of the effect of the various treatments upon pituitary gonadotropins was desired, a

[2] MER-25 was generously supplied by Dr. Robert H. McMaster of the Department of Medical Research of the William S. Merrell Company, Cincinnati, Ohio.

second series of the same four treatment groups was treated for two months to obtain representative groups of pituitary glands for gonadotropin assay. These hypophyses were pooled according to therapy, macerated, and suspended in alkaline saline. Their gonadotropin content was assayed in twenty-one day old female white mice which were injected subcutaneously twice per day for two and one half days with 0.2 cc. volumes of the concentrated solutions or appropriate dilutions. The mice were sacrificed twenty-four hours after the last injection, and the uteri were excised and weighed.

Gonadotropin Inhibition in Parabiotic Rats

Gonadotropin inhibiting effects of estrogen and MER-25, alone and in combination, were studied in thirty-one day old male albino rats which were castrated and parabiosed to intact female littermates according to the technique of Bunster and Meyer (3) as modified by Meyer and Hertz (20). Beginning with the day of operation, the male parabionts were injected subcutaneously once per day for ten days with either olive oil, 0.25 mcg. estradiol benzoate, 10.0 mg. MER-25, or a combination of estradiol and MER-25, each in 0.2 cc. volumes. These doses were selected because they inhibited the gonadotropin hypersecretion only partially. The animals were sacrificed on the eleventh day, and the ovaries of the intact female parabionts were removed and weighed. Pituitary glands were removed from the treated male partners and macerated for gonadotropin assays performed as described previously.

Spontaneous Rat Uterine Contractions in Vitro

To study the effect of MER-25 upon spontaneous rat uterine contractions *in vitro*, spayed female rats weighing 170–230 gm. were injected subcutaneously one week after surgery for three days with either 1.0 μg. estradiol per day, 5.0 mg. MER-25 per day, or a combination of both. The rats were sacrificed on the fourth day by a blow on the head. Uteri were removed promptly and longitudinal strips were suspended in muscle baths containing 50 ml. of Locke's Ringer's solution. Air was bubbled through the baths maintained at a constant temperature of 36.5 to 37° C, and contractions were recorded on a slow moving kymograph.

RESULTS

Pituitary Hypertrophy in Intact Adult Female Rats

Examination of the data revealed that organ weight changes in each of the treated groups had reached a plateau effect at the third month, and relatively little change occurred thereafter for the duration of the study. Figure 2 demonstrates this point for the pituitary gland weights of the rats injected with estradiol alone and estradiol plus MER-25 for each of the six autopsy periods. Each bar on the graph represents the average of results from four animals. Since organ weights were relatively consistent over the six month period of study with no persistent upward or downward trend, subsequent data are compared according to respective therapy regardless of its duration.

Table 1 presents the data on the effect of the various types of treatment upon the pituitary gland weights. Estradiol caused the anticipated pituitary hypertrophy, the average weight of the pituitaries being four times that of the controls. MER-25 did not induce hypertrophy by itself, but rather it significantly reduced the pituitary weights below control levels ($P = <0.01$). In combination therapy, MER-25 greatly antagonized both

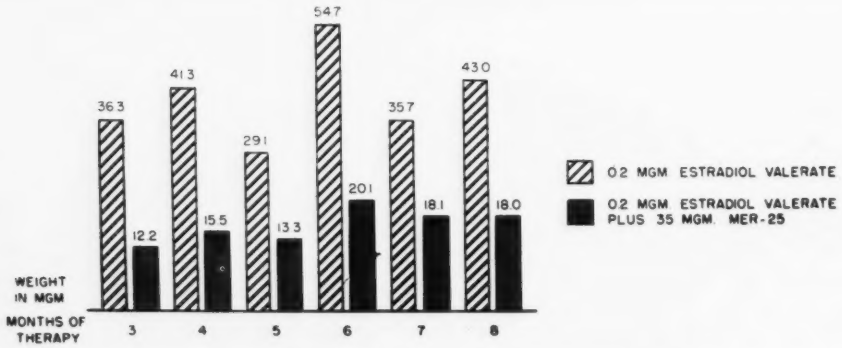


FIG. 2. The effect of MER-25 upon estradiol-induced pituitary hypertrophy in rats.

the gross and morphological effects of estradiol. The average weight of these hypophyses was 80% greater than that of control pituitaries, but less than one half the weight of the glands from estradiol-treated rats. Sections of typical pituitary glands obtained at autopsy are shown in Figure 3. The gland of an animal treated with MER-25 differs little from that of an untreated rat. The pituitary gland of a rat treated with estradiol exhibits degranulation and an increase in the number of chromophobes. The cellular morphology of a gland from an animal given combined estradiol and MER-25 therapy is intermediate between the responses to estradiol alone and MER-25 alone.

Ovarian weights of the rats treated with MER-25 were less than those of untreated control rats (Table 1). Estradiol, at the dose employed, caused an even greater diminution in ovarian size. The ovarian weight reduction by estradiol was relatively unaltered by the concomitant administration of MER-25 ($P = >0.05$).

Uterine weights (Table 1) were reduced below control levels by MER-25 which also inhibited the uterotropic effects of estradiol.

TABLE 1. THE EFFECT OF MER-25 UPON RATS CHRONICALLY TREATED WITH ESTRADIOL

Treatment	Dose per week	No. of rats	Mean organ weights ± standard error					
			Ovaries		Uterus		Pituitary	
			mg.	S.E.	mg.	S.E.	mg.	S.E.
None		8	59.2	±5.2	292.1	±25.0	9.6	±0.03
Estradiol Valerate	0.2 mg.	21	12.2	±1.3	365.4	±18.3	40.2	±3.5
MER-25	35 mg.	12	38.4	±4.0	154.2	±16.7	8.1	±0.05
Estradiol Valerate and MER-25	0.2 mg. 35 mg.	24	17.5	±1.9	256.9	± 7.2	16.2	±1.1

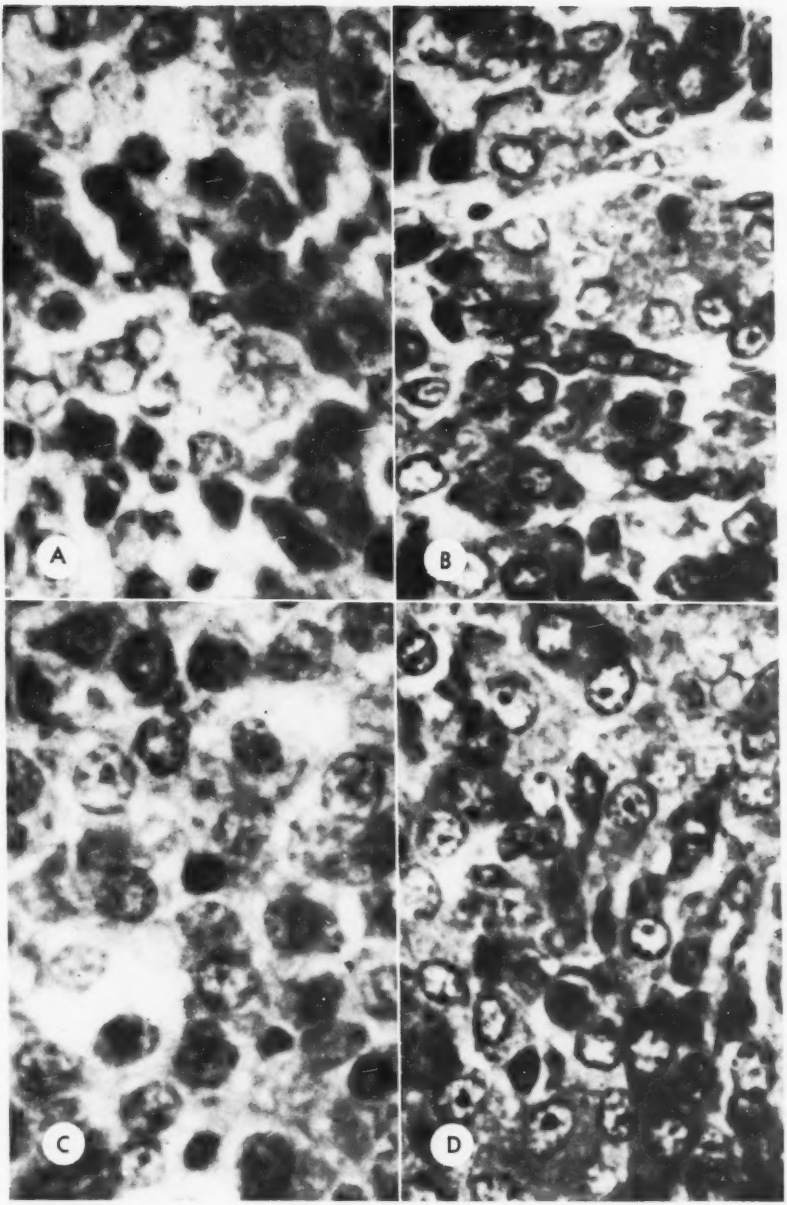


FIG. 3. Photomicrographs of anterior lobes of pituitary glands from chronically treated adult rats.

A. No treatment; normal rat pituitary gland. 10% formalin. Masson's trichrome 1200×.

B. 35 mg. MER-25 per week; this pituitary differs little from the gland of an untreated animal. 10% formalin. Hematoxylin and Eosin. 1200×.

C. 0.2 mg. estradiol valerate per week; note the degranulation and increase in the number of chromophobes. 10% formalin. Hematoxylin and Eosin. 1200×.

D. 0.2 mg. estradiol valerate plus 35 mg. MER-25 per week; the morphology is intermediate between the responses to estradiol alone and MER-25 alone. 10% formalin. Masson's trichrome. 1200×.

In the gonadotropin assays (Fig. 4) it was found that the pituitary glands of rats which had been injected with estradiol contained twenty mouse uterine units of gonadotropin. A mouse uterine unit of gonadotropin is the least amount of extract which, when administered to immature female mice in five injections over a period of two and one half days, will increase their uterine weights to twice that of an uninjected animal. Pituitaries from the MER-25 treated group contained 2.5 mouse units per gland as did those from the untreated rats. Hypophyses of animals which had been given combined estradiol and MER-25 therapy contained less than 2.5 mouse units of gonadotropin per gland. Since the assay method utilized serial 1:1 dilutions of pooled pituitary extracts, these results, per gland,

FIG. 4. Gonadotropin content of pituitary glands of rats treated with estradiol and MER-25. Mean values.

are intended to demonstrate only that the gonadotropin content of a pituitary gland from an estradiol-treated animal was much greater than the content of a control gland. A precise quantitation is not to be implied.

Gonadotropin Inhibition in Parabiotic Rats

At the doses used, estradiol benzoate and MER-25 each reduced the ovarian hypertrophy in the intact female parabionts to be slightly more than half the weights of the ovaries of the control parabionts (Table 2). Combined estradiol and MER-25 therapy appeared to reduce the ovarian weights even more than either drug did individually. However, this further decrease proved statistically insignificant ($P = >0.1$).

Gonadotropin assays performed in the same manner as with the pituitary glands from the intact single rats indicate that estradiol and MER-25, individually or together, increased the gonadotropin content of the pituitary glands of the castrate male parabionts above control levels (Table 2). However, because of variation in bioassay, rigid quantitation was not intended.

TABLE 2. THE EFFECT OF MER-25 UPON PARABIOTIC RATS TREATED WITH ESTRADIOL

Treatment	Dose per day	No. of pairs	Intact parabiont Ovarian weights Mean values ± standard error (mg.)	Gonadectomized parabiont Pituitary assays mouse units of gonadotropin per gland
Oil Medium		10	118.6 ± 7.9	2.0
Estradiol Benzoate	0.25 mcg.	14	65.5 ± 10.5	4.0
MER-25	10 mg.	16	64.5 ± 9.0	>4.0<8.0
Estradiol Benzoate and MER-25	0.25 mcg. / 10 mg.	17	46.4 ± 9.3	>4.0<8.0

Spontaneous Rat Uterine Contractions in Vitro

Since precise duplication of length and thickness of the muscle strips was impossible, it was deemed inadvisable to compare the responses on a numerical basis, but rather to view representative contraction patterns of the entire treatment groups (Fig. 5). It is known that there is considerable variation in the responses of individual uterine strips. However, pretreat

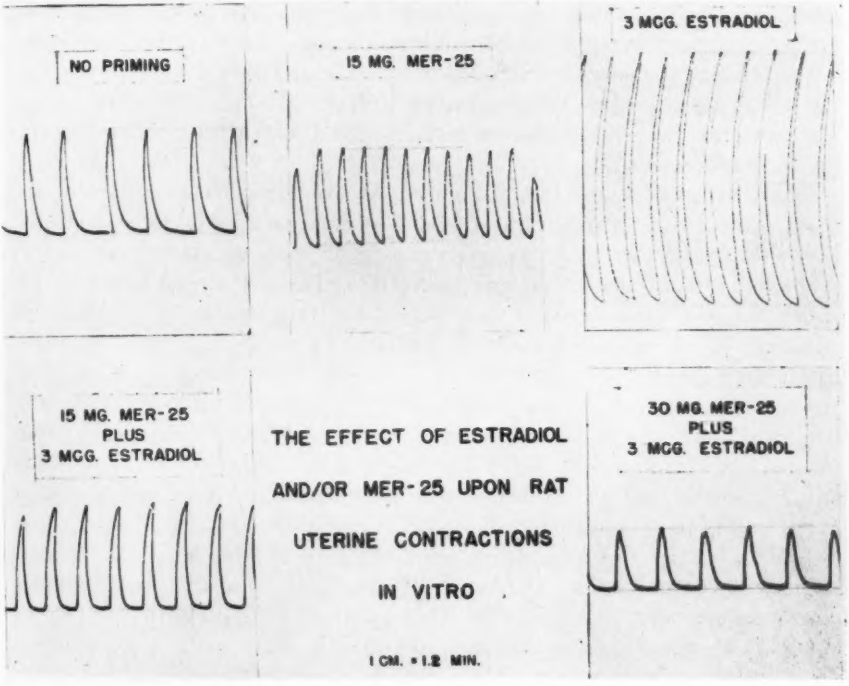


FIG. 5. The effect of estradiol and/or MER-25 upon rat uterine contractions *in vitro*. 1 cm. = 1.2 minutes.

ment with estradiol definitely enhanced uterine contractions in 58 of 62 strips. 15.0 mg. of MER-25 reduced the amplitude of control contractions in 16 of 19 strips and significantly antagonized the estradiol effect in all of the 17 strips from rats primed with both compounds. 30.0 mg. of MER-25 given in combination with 3.0 mcg. of estradiol further inhibited the amplitude and frequency of contractions.

DISCUSSION

Numerous investigators have described the effects of estrogen injections and pellet implantations upon the rat pituitary gland. The results have ranged from slight enlargement of the gland to distinct tumors as a result of one month to more than a year of treatment. Several have described the predominant cell type of the hypertrophic pituitary as chromophobic (8, 13, 14, 21, 22, 26, 27) and others as acidophilic (1, 6, 7, 11, 12, 17, 19). Since the chromophiles are often degranulated and hence cannot be accurately identified, classification of the hypertrophy as acidophilic has been based primarily upon the observation of associated mammary gland development and secretion. Microscopic examination in this study of the hypertrophic pituitaries obtained from rats chronically treated with estrogens showed the predominant cell type to be chromophobic. Neither the mammary gland hyperplasia nor milk secretion which have been observed with continuous estrogen stimulation was noted (1, 6, 19). This discrepancy may be due to differences in the method of estrogen administration (weekly subcutaneous injection of long acting esters of estradiol versus pellet implantation).

The increased gonadotropin content of the pituitaries of the treated animals over that of control animals noted by assay finds some confirmation in the literature (4, 10) while being apparently contrary to other reports (2, 16, 18). Nevertheless, the increased gonadotropin levels in this study suggest that estrogen exerts part of its gonadotropin inhibitory effects by blocking the release, rather than the production, of gonadotropin by the hypophysis.

MER-25 significantly antagonized the estrogen-induced pituitary hypertrophy. Despite its inherent estrogenic activity, it has been reported that the parent compound of MER-25, tri-*p*-anisyl chloroethylene, not only antagonizes this action of other estrogens but also does not cause pituitary hypertrophy in rats, although it itself is estrogenic (24, 25).

A decrease in the average ovarian weights of the MER-25 treated animals as compared with control weights suggests at least partial gonadotropin suppression by MER-25. This gonadotropin inhibitory effect of MER-25 is also demonstrated in the parabiotic rats where a decrease in the ovarian weights of the intact female parabionts was noted. When administered together, MER-25 did not antagonize the gonadotropin in-

hibition by estradiol. This apparent failure of MER-25 to antagonize the effect of estrogen upon gonadotropic activity might be anticipated since both compounds have been shown to act as gonadotropin inhibitors.

As in the chronic study with the single rats, gonadotropin assays of the pituitary glands from the castrate male parabionts indicate that estrogen apparently blocks the release of gonadotropin from the hypophysis. The mechanism by which MER-25 has its effect was not resolved by the gonadotropin assays since the pituitaries of parabionts which had been injected with MER-25 with or without estradiol were found to contain higher levels of gonadotropin while a lower gonadotropin content was found in the hypophyses of similarly treated single rats. This discrepancy may be due to differences in the gonadotropic potencies and hypophyseal-gonadal interrelationships of intact adult female and immature castrate male rats.

It is interesting to speculate as to the mechanism by which MER-25 reduced the uterine weights of untreated as well as estrogen-treated single intact rats. The drug may have a direct inhibitory effect upon the uterotropic activity of both endogenous and exogenous estrogens. It is also possible that MER-25 reduced uterine weights in the intact rats not only by antagonizing the effects of endogenous estrogens directly but also by its ability to inhibit gonadotropins and thereby secondarily reduce endogenous estrogenic production.

Further evidence of the ability of MER-25 to antagonize exogenous estrogens is manifested by administration of the drug *in vivo* and noting its inhibitory effect upon estrogen-induced uterine contractions *in vitro*. Since these rats were spayed, the possibility that the reduction of the amplitude of control contractions by MER-25 may be due to the inhibitory effect of endogenous estrogens emanating from the adrenal glands could not be ruled out. Further studies in gonadectomized and adrenalectomized animals would be necessary to confirm this hypothesis.

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THE EFFECT OF ADRENOCORTICAL STEROIDS ON THE RENAL AND EXTRA-RENAL RESPONSES OF THE DOMESTIC DUCK (*ANAS PLATYRHYNCHUS*) AFTER HYPERTONIC SALINE LOADING[1]

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ABSTRACT

All ducks showed an initial diuresis during the first hour after saline loading and at varying times during this phase the onset of nasal secretion occurred.

The total extra-renal secretion of sodium and potassium in response to a saline load was increased after treatment with adrenocortical steroids. The most potent hormone in this respect was aldosterone.

In the presence of exogenous adrenocortical steroids and ACTH the extra-renal response was independent of the rise in serum sodium as a result of the saline load.

The results are discussed in relation to similar studies with teleost fish.

ORAL and parenteral administration of sodium chloride to certain marine birds has been shown to stimulate an extra-renal excretion of electrolytes (1, 2, 3, 4). This response appears to be of an osmoregulatory nature since intravenous administration of sucrose will also trigger the mechanism (5). The site of extra-renal excretion has been shown to be associated with the paired supra-orbital glands which discharge their secretion via two ducts into the anterior nasal cavity (2, 6). This fluid may be collected as it appears at the external nares.

It has been demonstrated that the cormorant, seagull, penguin, albatross and domestic duck are all capable of extra-renal excretion of excess sodium choride in the form of this highly concentrated secretion (2, 3, 4, 5, 7). Since this mechanism permits the animal to remain in water balance it is of considerable survival value to the marine bird exposed to a high salt diet for prolonged periods.

The only other mechanism involving the excretion of large quantities of electrolytes is the kidney, and this, to a large extent, is under endocrine

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control. With this in mind therefore, an attempt was made in the present investigation to elucidate any possible endocrine control of the extra-renal processes in the saline loaded domestic duck. Earlier studies by Schmidt-Nielsen and his co-workers, indicated that parasympathetic stimulation of the supraorbital glands in the Herring Gull (*Larus argentatus*) was accompanied by nasal secretion (5). This would appear to be a vasomotor response but the active transport of sodium may be under hormonal control.

MATERIALS AND METHODS

All birds used throughout these experiments were 9–12 week old male domestic ducks of the Peking white variety. They were fed daily with commercial "duck grower food" and given an *ad libitum* supply of fresh water. The ten control birds and each experimental group of 5 animals were starved for 24 hours before use. A wing vein was cannulated and the bird was heparinized 1½ hours prior to the commencement of the experiment. A sheet of polyethylene was placed under the cloacal region and the bird was fastened in a prone position to a standard "dog board" by means of rubber loops over the wings and legs. The sheet was pulled down to form a funnel in the centre of the "dog board" and a hole was cut in the bottom of the funnel for urine collection.

Saline loading was by means of a flexible plastic tube connected to a syringe and 20 ml. of 20% NaCl was introduced directly into the glandular stomach (proventriculus). The time of saline loading was taken to be zero time for all experiments.

A control blood sample was taken from the cannulated wing vein prior to the saline loading and, subsequent to loading, samples were taken at ½ hourly intervals for the first 3 hours and at hourly intervals for the remaining experimental period. The blood was immediately centrifuged at 2° C and 5000 rpm for 5 minutes. Urine samples were collected and measured at 20 minute intervals after zero time. The time of onset of nasal secretion was recorded and collections were made over 45 minute periods thereafter.

At autopsy the adrenal glands were removed, cleaned of adjacent tissues and weighed on an analytical balance.

A Zeiss PF5 flame photometer was used for all sodium and potassium estimations of plasma, urine and nasal samples. All electrolyte estimations were expressed in milli-equivalents/litre.

The cortisol and cortexone treated birds received 5 mg. intramuscular doses 12 hours and 1½ hours prior to saline loading. The term cortexone rather than deoxycorticosterone is used throughout this text in accordance with the recommendations of Fieser and Fieser (8). Both these steroids were free alcohol preparations in stabilized suspension. ACTH (Acton X, Nordic Biochemicals Ltd.) was administered daily for 5 days prior to the experiment, each bird receiving 6 doses of 20 I.U. in isotonic saline. The *dl* aldosterone (CIBA) was in oil solution and two 250 μg. doses were given 1½ and zero hours before loading.

RESULTS

The dominant characteristic of these experiments was the diphasic response to a saline load. All birds showed an initial diuresis during the first hour after loading and at varying times during this phase the onset of nasal secretion occurred.

Renal phase Table 1

The control birds showed a mean total diuresis of 51.9 ± 3.2 cc. during the three 20-minute collection periods immediately after salt loading. The volume excreted during these periods progressively declined from 23.3 ± 4.8 cc. to 11.5 ± 4.2 cc. The disproportionate decline in the sodium content of these samples was reflected in the significant fall in concentration from 633.0 ± 70.4 to 248.0 ± 115.6 meq./l. ($P < .01$). Conversely the potassium concentration appeared to rise from 13.6 ± 2.8 to 24.7 ± 12.2 meq./l. in the same samples, but this change was not statistically significant.

Cortisol treatment did not reduce the initial diuresis, indeed the mean total volume was slightly greater. The total sodium output during this

TABLE 1. THE EFFECT OF ADRENOCORTICAL STEROIDS ON THE RENAL EXCRETION BY THE DOMESTIC DUCK FOLLOWING A SINGLE HYPERTONIC SALINE LOAD

	Urine sample	Volume in cc.	Total renal output in meq.			Urine concentration in meq./l.	
			Na^+	K^+	$Na^+:K^+$	Na^+	K^+
Control	1	23.3±4.8	14.8±6.2	0.27±0.02	49.2± 6.3	633.0± 70.4	13.6± 2.8
	2	17.1±4.3	7.7±1.8	0.17±0.06	52.1± 9.2	455.8± 34.8	11.0± 7.7
	3	11.5±4.2	2.4±1.6	0.27±0.23	46.3±16.5	248.0±115.6	24.7±12.2
	Total	51.9±3.2	24.9±2.9	0.71±0.23	47.4± 8.8	482.2± 24.1	12.7± 3.5
Cortisol	1	24.3±2.6	7.9±1.5	0.48±0.09	18.9± 2.8	327.0± 31.9	16.1± 2.9
	2	23.3±0.9	6.5±0.7	0.26±0.002	24.8± 2.7	265.6± 17.3	11.5± 1.1
	3	12.2±6.4	2.2±1.8	0.25±0.10	7.3± 3.8	179.8± 67.4	36.7±14.9
	Total	59.8±7.9	16.6±2.4	0.99±0.18	17.1± 2.1	278.1± 23.2	17.8± 2.9
Cortexone	1	30.3±5.3	20.6±3.4	0.33±0.05	62.1± 1.3	682.2± 83.4	10.9± 0.9
	2	8.5±3.2	3.0±1.1	0.06±0.002	52.4± 4.2	358.3± 3.3	7.1± 0.4
	3	9.9±3.7	1.5±1.2	0.66±0.24	2.1± 1.4	158.0±109.6	61.5±12.6
	Total	48.7±5.8	25.1±3.1	1.05±0.26	34.2± 8.4	520.3± 15.2	11.8± 5.3
Aldosterone	1	11.6±3.0	4.6±1.6	0.19±0.04	25.9± 6.8	389.1± 67.6	19.3± 5.9
	2*	2.5	1.2	0.01	23.3	467.8	5.8
	3	—	—	—	—	—	—
	Total	14.1±4.9	5.8±2.4	0.20±0.05	28.8± 9.7	361.1± 68.4	18.3± 6.2
ACTH	1	35.6±2.9	20.3±2.5	0.43±0.004	47.0± 5.5	576.0± 49.2	12.7± 1.4
	2	18.6±4.3	7.3±2.1	0.15±0.01	51.5± 9.9	394.8± 60.6	7.8± 0.3
	3	5.9±3.2	2.0±1.1	0.04±0.02	71.2±21.3	380.0± 18.9	5.9± 1.6
	Total	60.1±6.8	29.6±2.7	0.62±0.02	49.1± 3.3	503.9± 18.9	10.3± 0.5

* One urine sample from one individual.

diuresis was, however, less than the control value due to the fact that the first sample contained 7.9 ± 1.5 meq. in 24.3 ± 2.6 cc. whereas the first control sample contained 14.8 ± 6.2 meq. in 23.3 ± 4.8 cc. A trend toward diminishing sodium concentration of the individual samples was also apparent after cortisol treatment. As in the case of the control birds the pattern of potassium excretion was not so clear-cut, and the total output of 0.99 ± 0.18 meq. was not significantly greater than the control value of 0.71 ± 0.23 meq. Examination of the Na/K ratios showed a very significant decline compared to the corresponding control values ($P < .01$).

Treatment with cortexone did not significantly alter the total diuresis although there was a 70% reduction in volume between the first and third samples compared with a 50% decline in the controls. Similarly, there was no significant change in the total sodium excreted when compared to the controls but there was a significant fall from 20.6 ± 3.4 meq. in the first

sample to 1.5 ±1.2 meq. in the third sample ($P < .01$). This represented a 92% reduction compared to 83% in the controls. Therefore, although there was an increased reabsorption of water under the influence of cortexone there did not appear to be a commensurate increase in the tubular reabsorption of sodium. Comparison of the potassium content of the first and third urine samples showed an apparent rise from 0.33 ±0.05 meq. to 0.66 ±0.24 meq. but this increase was not significant. This same trend was reflected in the urine concentrations of sodium and potassium and in the Na/K ratios.

There was a marked reduction in the diuretic phase of the aldosterone treated birds. Only one bird urinated during the second sample period and none during the third. Consequently the total volume excreted (14.1 ±4.9 cc.) was significantly lower than any other group ($P < .001$). The total sodium excreted was 5.8 ±2.4 meq. in a mean volume of 14.1 ±4.9 cc. This concentration of 361.1 ±68.4 represented a considerable decline in renal sodium excretion when compared to the control birds. Although the total potassium excreted was significantly less than the control value (0.20 ±0.05 Vs. 0.71 ±0.23 meq.) there was, however, a trend towards an increase in the potassium concentrations of these urines (12.7 ±3.5 Vs. 18.3 ±6.2 meq./l.).

As in the case of cortisol treatment, ACTH slightly increased the total volume of urine excreted during the first hour, but although these volumes were virtually identical (51.9 V 60.1 cc.) the ACTH group excreted approximately 80% more sodium than the cortisol group and this was reflected in the sodium concentrations of the successive urine samples. Indeed, the total sodium excreted after treatment with ACTH (29.6 ±2.7 meq.) appeared higher than both the cortisol (16.6 ±2.4 meq.) and the control (24.9 ±2.9 meq.) values, but the difference was only significant when compared to cortisol ($P < .01$). The urine sodium concentrations, however, were in no case higher than the corresponding control values. The potassium output in the three urine samples declined as in the case of the control and cortisol groups.

Extra-renal phase Table 2, Figure 1

The initial rate of nasal secretion for the control birds during the first 45 minute collection period was 1.58 ±0.42 cc. and this rate of output increased to a maximum of 4.95 ±0.56 cc. during the third period. The subsequent decline in output resulted in complete cessation in three of the birds during the final period i.e., 450 to 495 minutes after loading. The concentrations of sodium and potassium in the nasal fluid did not differ between successive samples and the total outputs in the 28.9 ±3.5 cc. discharged were 17.1 ±1.6 and 0.44 ±0.06 meq. respectively indicating mean concentrations of 602.6 ±13.9 and 17.3 ±0.49 meq./l. The ratios of extra-renal to renal excretion of sodium and potassium were 0.85 ±0.22 and 0.94 ±0.30 respectively.

TABLE 2. THE EFFECT OF ADRENOCORTICAL STEROIDS ON THE EXTRA-RENAL OUTPUT OF SODIUM AND POTASSIUM BY THE DOMESTIC DUCK FOLLOWING A SINGLE HYPERTONIC SALINE LOAD

	Total Output					Concentrations			Initial rates of output			Nasal: Urine ratio		
Group	Body wt., kg.	Volume, cc.	Na^+ meq.	K^+ meq.	$Na^+:K^+$ ratio	Na^+ meq./liter	K^+ meq./liter	$Na^+:K^+$ ratio	Volume, ccs./hr.	Na^+ meq./hr.	K^+ meq./hr.	Volume	Na^+	K^+
Control	2.54 ±0.04	28.9 ±3.5	17.1 ±1.6	0.44 ±0.06	36.8 ±2.8	602.6 ±13.9	17.3 ±0.49	36.8 ±3.9	2.1 ±0.8	1.16 ±0.4	0.031 ±0.01	0.35 ±0.06	0.85 ±0.22	0.94 ±0.30
Cortisol	2.66 ±0.09	59.0** ±5.1	31.3** ±2.4	0.89** ±0.06	35.6 ±0.8	534.0* ±14.8	15.2** ±0.40	35.2 ±0.4	7.1** ±1.1	3.63** ±0.4	0.093** ±0.02	1.07** ±0.10	2.08** ±0.29	0.97 ±0.17
Cortexone	2.74 ±0.11	54.6** ±4.6	27.9** ±2.8	0.92** ±0.09	31.2 ±2.8	511.2** ±11.9	16.8 ±1.10	26.9* ±2.1	6.5** ±0.8	3.13** ±0.4	0.092** ±0.004	1.25** ±0.26	1.35 ±0.13	1.62 ±0.73
Aldosterone	2.46 ±0.05	82.1** ±3.8	45.9** ±2.2	1.08** ±0.04	42.8 ±0.8	560.4* ±13.8	13.2** ±0.56	42.8 ±0.8	6.7* ±2.7	3.70 ±1.4	0.084 ±0.03	5.59** ±1.22	11.06* ±3.70	4.47** ±0.48
ACTH	2.42 ±0.06	40.3* ±4.2	21.5 ±1.7	0.66 ±0.10	35.7 ±1.6	541.9* ±19.8	15.3* ±0.81	35.7 ±1.6	8.2** ±1.9	3.65** ±0.6	0.106** ±0.02	0.76* ±0.18	0.76 ±0.10	1.05 ±0.15

* P = <.05 with rsspect to corresponding control value.
** P = <.01 with respect to corresponding control value.

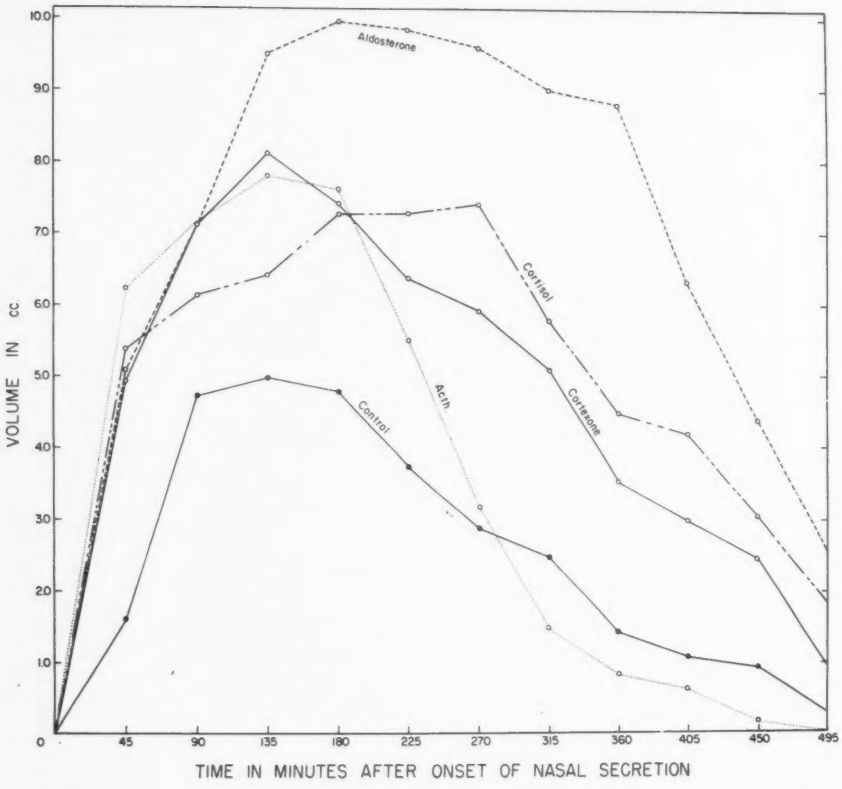


FIG. 1. The volumes of nasal discharge collected from the domestic duck during successive 45 minute periods following the administration of a single hypertonic saline load and the effect of pre-treatment with cortisol, cortexone, aldosterone and ACTH on this response.

After cortisol treatment there was a significant increase in the initial rate of nasal discharge (1.58 ± 0.42 Vs. 5.34 ± 0.84 cc./45 min. collection period). This increase continued up to a maximum between 225 and 270 minutes after loading when the output was 7.40 ± 0.99 cc., and subsequently a decline occurred which continued throughout the remainder of the experiment. None of the cortisol treated birds ceased to drip during the experimental period and the mean total output of 59.0 ± 5.1 cc. was significantly greater than the corresponding control volume of 28.9 ± 3.5 cc. ($P < .01$). The sodium concentration of the nasal fluid, however, was lower than the control and this decrease was significant at the 5% level. On the other hand, due to the enhanced nasal volume, the total sodium output was significantly higher in the cortisol treated ducks ($P < .01$). The potassium concentration was similar to the control value and represented ap-

proximately 3% of the sodium concentration. The extra-renal: renal ratio for sodium was 2.08 ± 0.29, which reflected the simultaneous extra-renal enhancement and renal depression of sodium ouput. The value for potassium did not differ significantly from the controls (0.97 ± 0.17 Vs. 0.94 ± 0.30).

The effect of cortexone on the nasal secretion after salt loading appeared to be less than that observed for cortisol. Nevertheless, the mean output during the first 45 minutes (4.92 ± 0.62 cc.) and the mean total volume secreted (54.6 ± 4.6 cc.) were significantly higher than the control values ($P < .01$). The maximum rate of nasal secretion was between 90 and 135 minutes after loading which coincided with the control birds but preceded the cortisol treated animals. As in the case of cortisol, the sodium concentration of the nasal discharge was significantly lower than the controls ($P < .01$) although the potassium concentration was the same, but due to the enhanced volume secreted the total output of sodium and potassium were significantly greater than the controls ($P < .01$).

Treatment with aldosterone resulted in a nasal secretion of 5.04 ± 2.05 cc. during the first 45 minute collection period which, during the period from 135 to 180 minutes after loading, increased to 9.94 ± 1.67 cc. This individual volume and the total nasal output of 82.1 ± 3.8 cc. were the highest volumes recorded in any of these experiments. Again the mean sodium concentration of the nasal secretion was significantly lower than the control birds ($P < .05$) but the total extra-renal sodium output (45.9 ± 2.2 meq.) was higher due to the increased volume ($P < .01$). Similarly the total potassium output was significantly higher in spite of a decline in the concentration from 17.3 ± 0.49 to 13.2 ± 0.56 meq./l. compared to the controls ($P < .01$). Examination of the nasal:urine ratios showed a value for sodium which was twice that observed with respect to volume and potassium. This was at least in part due to the high renal tubular reabsorption of sodium and the simultaneous enhancement of nasal secretion. Cortisol was the only other hormone which brought about this disproportionate ratio for sodium compared to the ratios for volume and potassium.

ACTH induced a significant increase in the total nasal volume secreted (40.3 ± 4.2 cc.) and the initial rate of output was the highest recorded (6.18 ± 1.4 cc./45 minutes). The period of response, however, was somewhat shorter than the control birds since the decline in secretion was more rapid. The nasal sodium concentration of 541.9 ± 19.8 meq./l. was significantly lower than the controls ($P < .05$) and there was consequently a disproportionately lower increase in the total extra renal sodium secreted compared to the controls (21.5 ± 1.7 V $17,1 \pm 1.6$ meq.).

Serum electrolyte changes Table 3 and Figure 2

During the first hour after saline loading there was a steady rise in the serum sodium concentration of all groups and a plateau was attained after ninety minutes. The initial rate of increase in serum sodium concentration

TABLE 3. THE EFFECT OF ADRENOCORTICAL STEROIDS ON THE SERUM ELECTROLYTE CHANGES AND THE ONSET OF NASAL DISCHARGE BY THE DOMESTIC DUCK IN RESPONSE TO A SINGLE HYPERTONIC SALINE LOAD

Group	Time of onset of nasal secretion mins.	"P" value	Initial rise in serum Na^+ meq. /l./hr.	"P" value	Serum Na^+ at onset of nasal secretion meq. /l.	"P" value
Controls	52.8±11.3	—	28.7±2.3	—	160.6±2.5	—
Cortisol	16.0± 6.5	<.02 >.01	31.8±2.9	>.4	153.9±2.4	<.1 >.05
Cortexone	24.0± 6.5	<.05 >.10	35.6±3.3	<.2 >.1	159.2±2.6	>.8
Aldosterone	14.0± 3.3	<.01 >.001	30.0±5.3	>.8	152.0±1.2	<.01 >.001
ACTH	11.4± 1.9	<.01 >.001	34.6±2.6	<.2 >.1	151.8±1.6	<.01 >.001

did not differ significantly in any group from the control animals but the onset of nasal secretion was significantly earlier in birds after treatment with cortisol, cortexone, aldosterone and ACTH.

Examination of the serum sodium concentrations at these times of onset

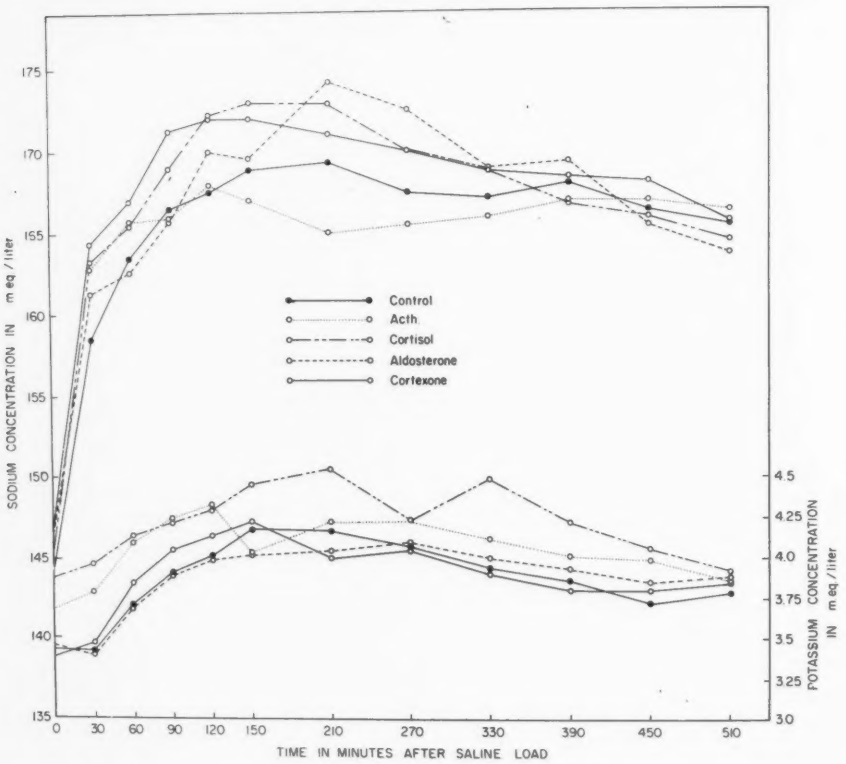


FIG. 2. The progressive changes in the serum electrolyte composition of the salt loaded domestic duck after treatment with adrenocortical steroids and ACTH.

indicated that in the case of aldosterone and ACTH, nasal secretion occurred at 152.0 ± 1.2 and 151.8 ± 1.6 meq./l. respectively. These were significantly lower concentrations than the value of 160.6 ± 2.5 meq./l. ($P < .01$) in the control birds. The serum sodium concentration at the time of onset of nasal secretion in the cortisol treated birds was 153.9 ± 2.4 meq./l. and was significantly lower than the control value at better than the 10% level. The maximum serum sodium concentration was attained in all except the aldosterone group between 120 and 150 minutes after loading and there was no significant difference between this value for any of the experimental groups and the control birds. During the remainder of the nasal secretory phase there was a gradual decline in the serum sodium concentration of all animals, however, in no group did the serum sodium concentration return to the pre-loading values and the terminal

TABLE IV. THE EFFECT OF INTRAMUSCULAR ADMINISTRATION OF ACTH (6 DAILY DOSES OF 20 I.U.) ON THE ADRENAL GLAND WEIGHTS OF THE DOMESTIC DUCK

Group	Body wt. kg.	Left adrenal, mg.	Right Adrenal, mg.	Total adrenal, mg.	Adrenal wt. / Body wt. mg. %
Control	2.54 ± 0.04	80.8 ± 3.2	76.7 ± 4.0	157.4 ± 7.1	6.19 ± 0.26
ACTH	2.42 ± 0.06	106.0 ± 12.3	98.9* ± 6.7	204.9* ± 12.1	8.48** ± 0.48

* "p" value < .05 with respect to corresponding control value.
** "p" value < .01 with respect to corresponding control value.

concentration was in all cases higher than the threshold level required to initiate nasal secretion.

The serum potassium concentrations similarly rose in all groups during the first 120 minutes after loading and there was a steady decline throughout the remainder of the period. The initial rise in potassium was delayed some thirty minutes behind the rise in serum sodium.

Adrenal weight changes Table 4

Daily treatment with 20 I.U. of ACTH during the five days prior to experimentation induced a 37% increase in the adrenal/body weight ratio. There was also a significant increase in the absolute adrenal weights from a control value of 157.4 ± 7.1 mg. to 204.9 ± 12.1 mg. after ACTH treatment ($P < .05$).

DISCUSSION

The initial diuresis followed by a period of extra-renal sodium excretion in response to a saline load has also been reported by Scothorne (4) for the Aylesbury variety of the domestic duck. Schmidt-Nielsen also observed a similar diphasic response in the cormorant (*Phalacrocorax auritus*) after the oral administration of sea water (3).

A clue to the controlling factors associated with the extra-renal excre-

tory mechanism in these birds was indicated by the renal electrolyte excretion pattern of the control group in the present work. There was a progressive decline in the renal sodium output coincident with the onset of nasal secretion. Also the concentrations of sodium and potassium in the successive urine samples were compatible with an increased adrenocortical activity. Furthermore, in the birds treated with exogenous adrenocortical steroids and ACTH there was a direct correlation between the effectiveness of these hormones on the renal excretory pattern and the nasal secretory activity which ensued.

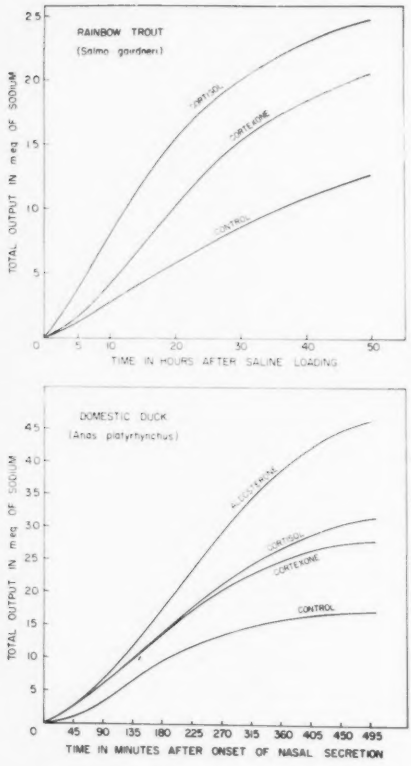
It may be suggested that the enhanced extra-renal secretion was a compensatory mechanism to offset the reduced renal excretion under adrenocortical influence. This, however, was clearly not the case with cortexone where the effect on renal sodium was not very apparent in terms of total output but nevertheless there was a significant increase in the nasal secretion. Previous workers (5) have suggested that the triggering mechanism associated with the onset of nasal secretion is of an osmoregulatory nature and they have shown that the infusion of hypertonic sucrose will elicit this phenomenon. The present work also suggested that some such osmoregulatory stimulus was necessary inasmuch as the onset of nasal secretion was always preceded by a rise in serum sodium. This initial rate of increase in serum sodium was identical in all experimental and control groups but in the case of aldosterone, ACTH, and cortisol the onset of nasal secretion appeared independent of the presumed osmoregulatory stimulus since it commenced when the sodium level was significantly lower than in the control animals. Furthermore, the initiation of nasal secretion in each experimental group preceded the time at which this event occurred in the controls. This would suggest that the increased time-lag in the controls marks a period during which the endogenous mobilization of adrenal steroids takes place to the threshold level necessary for the precipitation of nasal secretion. In the experimental groups no such lag occurs since sufficient levels of exogenous hormones are circulating at the time of loading. In other words the nasal gland had been previously "primed" and was galvanized into action at a lower level of osmotic stimulation. Certain preliminary studies substantiated this thesis since it was observed that a spontaneous nasal secretion could be induced in normal unloaded ducks which had received intramuscular doses of cortisol and 9α fluorocortisol. It is interesting in this respect to note that Frings and Frings (7) working with the albatross observed that ". . . excitement or stress caused the nasal gland to become active when ordinarily it would not be." Recent work in this laboratory involving the adrenalectomy of domestic ducks prior to salt loading showed a complete obliteration of the extra-renal response.

The results obtained from the ACTH treated ducks were somewhat disappointing for although there was a 37% increase in total adrenal gland

weight, and there was no reason to suspect that this was a medullary response, the increase in total nasal secretion was the least significant recorded. It is pertinent to note, however, that the endogenous adrenocortical activity in these birds brought about the highest initial response in terms of the nasal secretory rate. The relatively large doses of ACTH used may have exhausted or reduced the potential of the adrenocortical tissue to respond to the osmotic stimulus. Phillips and Chester Jones (9) reported a 78% lowering of the corticosterone secretion by the capon following treatment with high doses of ACTH. On the other hand these workers noted an increase in the levels of cortisol and aldosterone secretion. Such an increased secretion of cortisol and aldosterone may have accounted for the enhanced initial rate of nasal secretion observed in the present work. However, since the role of corticosterone in this mechanism is at present not known any relationship between the total nasal output after ACTH treatment and a possible lowering of the circulating levels of corticosterone cannot be made.

Further effects of the adrenocortical steroids on the extra-renal response included a three-fold increase in the initial rate of secretion and this rate was sustained in some cases (*cf.* cortisol and aldosterone) for a longer period than in the control ducks. This resulted in the increased extra-renal loss of sodium although the concentration of the nasal secretion in none of the hormone treated groups was as high as the controls, and was very significantly lower in the case of cortisol and cortexone. This suggested the possiblity of some other factor or factors involved in the extra-renal response.

With the possible exception of the Amphibia, the periodic exposure to a marine environment is found in all vertebrate classes. The anadromous teleosts, for example, are able to withstand wide variations in the tonicity of their environment, indeed, the salmon and the steelhead trout migrate from fresh water to sea water and back in the natural course of their life histories. Consideration of the problems which confront an animal exposed to a hypertonic sea water environment or to a long term diet containing sea water indicate that the animal must conserve water and simultaneously excrete sodium, potassium and chloride which are the major ionic constituents of sea water. The secretion of a nasal discharge containing sodium at a level higher than 100% sea water (600 V 470 meq./l.) and with an Na/K ratio well within the limits of sea water (36 ± 3.9 V 48.3) is clearly a mechanism whereby sodium and potassium may be lost without the excessive excretion of water. Just as in the case of the marine bird on a sea water diet, the salmonid teleost fish must also excrete excess quantities of sodium and potassium without the loss of commensurate volumes of water in order to survive a transfer from a hypotonic to a hypertonic environment. Comparison of the cummulative output of sodium from saline loaded trout in the presence and absence of certain adrenocortical



FIGS. 3a–b. Comparison of the cumulative extra-renal outputs of sodium from saline loaded fresh water trout (Salmo gairdneri) and the saline loaded domestic duck (Anas platyrhynchus). (Adapted from Holmes (10), Chester Jones, *et al.* (11) and the present work).

steroids with the cummulative output of sodium from similarly treated ducks showed that, although the quantities of sodium and the time relationships are not strictly comparable, the same basic pattern of excretion was observed in response to these steroids (10, 11. Fig. 3a and b).

Even though the present experiments were of a chronic nature and the total volumes of water excreted by the birds were greater than the intake, there was probably some antidiuretic factor operating, since the renal phase of excretion ended so abruptly during the first hour. Changes in the neurosecretory activity of the hypothalamus have been observed in fish during the first 4 hours after transfer to sea water (12, 13, 14) and the possibility, therefore, of a coincidental antidiuretic secretion of hypothalamic origin in response to a sline load in the marine bird may prove to be profitable field for further investigation.

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THE EFFECT OF HYDROCORTISONE AND PROGESTERONE ON THE ELECTROLYTE ACTION EXHIBITED BY DESOXYCORTICOSTERONE IN THE ADRENALECTOMIZED RAT[1]

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ABSTRACT

The influence of hydrocortisone and progesterone on the action of desoxycorticosterone (DOC) on sodium and potassium excretion was investigated in adrenalectomized rats. Hydrocortisone at low levels facilitated sodium excretion and at all levels tested facilitated potassium excretion. Progesterone did not elicit any effect on electrolyte excretion. When administered concomitantly with DOC, increasing dosages of hydrocortisone and progesterone induced sodium excretion even in the presence of DOC. By comparison of dosages hydrocortisone was shown to be 40 times as active as progesterone in bringing about 50% block of DOC effect. The simultaneous administration of DOC and hydrocortisone produced an increased excretion of potassium; this effect was not observed when DOC and progesterone were administered concomitantly.

THE complex interrelation of adrenal cortical steroids on the regulation of electrolyte metabolism is not well understood. Previous workers (1–2) have demonstrated an antagonism between several steroids such as cortisone, hydrocortisone, and progesterone on the action of desoxycorticosterone (DOC) and aldosterone on sodium and potassium excretion when tested in adrenalectomized animals. Suggestive evidence of this type of action has been accumulated in humans (3–8).

In order to study the effect of hydrocortisone and progesterone on the action of DOC in the adrenalectomized rat, the following experiments were undertaken.

METHOD

Mature Sprague Dawley male rats, weighing 135 to 145 grams were used in all experiments. The rats were adapted to a constant temperature (78° F) and humidity (50%) for 72 hours prior to adrenalectomy and remained on a diet of Purina fox chow and tap water.

1. At the start of the experiment (0 hr.) bilateral adrenalectomy was performed and

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the animals were maintained on 0.9% sodium chloride solution for 18–20 hours. Then, saline was substituted for tap water for 24 hours.

2. At the 48th hour, animals were lightly etherized, a sodium load of 3.0 cc. of 0.9% of sodium chloride was given subcutaneously. Simultaneously the rats received half the dose of the various steroids tested in 0.20 cc. of olive oil subcutaneously. Control animals were given 0.20 cc. of olive oil by the same route.

3. At the 51st hour, the remaining half dose of the various steroids under investigation was given subcutaneously in 0.20 cc. of olive oil. Control animals were given 0.20 cc. of olive oil by the same route.

4. At the 52nd hour, animals were lightly etherized and a ligature was placed on the urethra.

5. Animals were deprived of food but water was given at steps 2, 3, and 4.

6. At the 54th hour, the animals were killed by etherization, a hemostat was placed on the neck of the bladder and the entire urinary bladder and its contents removed.

The urine was recovered as in the procedure described by Kagawa *et al.* (9) and analyzed for sodium and potassium content after suitable dilution by flame photometry using an Internal Lithium Standard.

The experimental design was as follows: animals were divided into different groups; each group received various dosages of DOC, hydrocortisone, progesterone, as well as hydrocortisone and/or progesterone with varying dosages of DOC. In each group the dosage of the steroid or combination of steroids assigned to it was given to each animal separately and/or simultaneously (at two different sites).

Sodium retention or excretion was calculated as the percentage of sodium retained or excreted relative to values observed in control animals. DOC inhibition designated also as per cent block, was calculated as the percentage inhibition of the sodium retention induced by the same dose of DOC simultaneously tested according to the following formula:

$$\frac{[\text{mg. Na in DOC}+\text{Cpd tested group}]-[\text{mg. Na in DOC (alone) group}]}{[\text{mg. Na in control group}]-[\text{mg. Na in DOC (alone) group}]}\times 100$$

RESULTS

Effect of Hydrocortisone on the Electrolyte Action of DOC

Tables 1 and 2 show the effect of DOC and hydrocortisone when administered alone and/or simultaneously (at two different sites). These tables represent composite data obtained from 29 assays.

TABLE 1. THE EFFECT OF DOC AND HYDROCORTISONE ON ELECTROLYTE EXCRETION IN ADRENALECTOMIZED RATS

Material administered	Dose, μg	Total bladder content				No. of animals
		Na, mg. ± SE	% of the control (P)	K, mg. ± SE	% of the control (P)	
Olive Oil	0	2.83 ± 0.18	100	3.75 ± 0.16	100	67
DOC	6	1.15 ± 0.15	41 (<0.01)	4.25 ± 0.26	113 (<0.01)	43
	12	0.81 ± 0.14	29 (<0.01)	4.67 ± 0.28	125 (0.01)	29
	50	0.25 ± 0.01	9 (<0.01)	5.20 ± 0.52	139 (<0.01)	15
	100	0.22 ± 0.06	8 (<0.01)	4.71 ± 0.31	126 (<0.01)	22
	200	0.25 ± 0.03	9 (<0.01)	5.53 ± 0.58	147 (>0.1<0.5)	22
Hydrocortisone	12	2.64 ± 0.36	93 (>0.5)	3.19 ± 0.36	85 (<0.01)	12
	24	2.57 ± 0.48	91 (>0.5)	2.26 ± 0.41	60 (>0.5)	11
	48	4.27 ± 0.59	150 (>0.02<0.05)	3.93 ± 0.36	105	10
	100	3.24 ± 0.34	114 (>0.1<0.5)	4.65 ± 0.28	124 (<0.01)	19
	200	4.65 ± 0.42	164 (<0.01)	5.52 ± 0.46	147 (<0.01)	19
	500	3.46 ± 0.26	122 (0.05)	6.24 ± 0.54	166 (<0.01)	25
	1000	2.41 ± 0.16	85 (>0.05<0.1)	6.39 ± 0.52	170 (<0.01)	15

TABLE 2. THE EFFECT OF DOC AND HYDROCORTISONE ADMINISTERED SIMULTANEOUSLY ON ELECTROLYTE EXCRETION IN THE ADRENALECTOMIZED RAT

Material administered		Ratio hydrocortisone DOC	Total bladder content			Inhibition of DOC effect† on sodium	No. of animals
Hydrocortisone‡ μg	DOC, μg		Na, mg. ± SE	Na excretion, P*	K, mg. ± SE		
12	6	2/1	1.11 ± 0.26		4.26 ± 0.38	—	12
24	6	4/1	1.06 ± 0.29		4.69 ± 0.76	—	10
48	6	8/1	2.12 ± 0.29	<0.01	5.52 ± 0.73	58	12
100	6	17/1	3.08 ± 0.50	<0.01	5.70 ± 0.52	115	12
100	12	8/1	1.34 ± 0.26	>0.05 <0.1	6.18 ± 0.40	26	11
100	100	1/1	0.85 ± 0.24	>0.05 <0.1	7.03 ± 0.65	24	10
100	200	1/2	0.39 ± 0.10		6.03 ± 0.53	5	4
200	6	33/1	4.13 ± 0.79	<0.01	6.32 ± 0.89	177	7
200	12	17/1	5.03 ± 1.11	<0.01	5.56 ± 1.14	209	7
200	50	4/1	2.07 ± 0.57	<0.01	9.64 ± 0.95	70	12
200	100	2/1	0.65 ± 0.15	>0.05 <0.1	9.44 ± 0.55	16	13
200	200	1/1	0.81 ± 0.36	>0.05 <0.1	8.37 ± 0.29	22	12
500	6	83/1	2.85 ± 0.31	<0.01	6.72 ± 0.25	101	12
500	12	42/1	1.74 ± 0.27	<0.01	6.58 ± 0.67	46	5
500	50	10/1	2.65 ± 0.55	<0.01	7.18 ± 0.58	93	10
500	100	5/1	1.52 ± 0.29	<0.01	7.73 ± 0.31	50	10
500	200	2.5/1	1.48 ± 0.24	<0.01	7.50 ± 0.29	48	5
1000	6	167/1	2.64 ± 0.24	<0.01	6.46 ± 0.40	89	9
1000	12	83/1	2.24 ± 0.30	<0.01	6.93 ± 0.71	71	8
1000	50	20/1	1.44 ± 0.54	>0.02 <0.05	6.81 ± 0.95	46	5
1000	100	10/1	2.15 ± 0.78	0.02	6.65 ± 1.27	74	4
1000	200	5/1	3.75 ± 0.95	<0.01	8.07 ± 0.62	136	5

* Compared with excretion values corresponding to each dose of DOC individually tested presented in Table 1.
† Sodium excretion values corresponding to each dose of DOC individually tested presented in Table 1.

Sodium retention and potassium excretion were observed with dosages of DOC ranging from 6 to 200 μg. The sodium retaining effect followed a linear response from 6 to 50 μg. The response demonstrated a plateau from 50 to 200 μg dosage levels. Hydrocortisone showed sodium excreting effects above control values at dosages ranging from 48 to 500 μg. This effect was statistically significant at 48 and 200 μg ($P \leq 0.05$). Significant potassium excretion above the level observed in control animals was observed at dosages ranging from 100 to 1000 μg ($P < 0.01$).

Dosages of 12 and 24 μg of hydrocortisone were not effective in blocking the sodium retaining properties of 6 μg of DOC. When 48 μg of hydrocortisone were administered simultaneously with 6 μg of DOC 58% block occurred ($P < 0.01$). When 100 μg of hydrocortisone were used, a ratio hydrocortisone/DOC of 17/1 brought about complete inhibition of DOC effect ($P < 0.01$). With the same dose of hydrocortisone but with ratios of 8/1 and 1/1 the blocking effect was not satistically significant. With a ratio of 1/2 no inhibition of DOC effect occurred.

With 200 μg of hydrocortisone the following ratios hydrocortisone/DOC were used: 33/1; 17/1; 4/1; 2/1 and 1/1. Ratios of 33/1 and 17/1 produced complete block ($P < 0.01$); 70% inhibition occurred with a ratio of 4/1 ($P < 0.01$). Ratios of 2/1 and 1/1 were ineffective.

With 500 μg of hydrocortisone the ratios hydrocortisone/DOC used were: 83/1; 42/1; 10/1; 5/1 and 2.5/1. Complete block was obtained with a ratio of 83/1 ($P < 0.01$). Ratios of 42/1; 10/1; 5/1 and 2.5/1 produced 46 to 93% inhibition of DOC effect ($P < 0.01$).

With 1000 μg of hydrocortisone the ratios hydrocortisone/DOC used were: 167/1; 83/1; 20/1; 10/1 and 5/1. These ratios brought about 46

to 89% block ($P \leq 0.05$) except for a ratio of 5/1 which produced complete inhibition of DOC effect ($P < 0.01$).

The simultaneous administration of DOC and hydrocortisone resulted in a greater potassium excretion than that obtained with DOC alone, which was significant in all cases ($P \leq 0.02$) except when hydrocortisone and DOC were given at dosages of 100 and 6 μg and 200 and 12 μg of each compound respectively ($P > 0.1 < 0.5$).

In an effort to investigate if the action of hydrocortisone would be reversed with an excess of DOC, large dosages of DOC were administered simultaneously with hydrocortisone. Results are shown in Table 3.

Inhibition of the sodium retaining properties of 600 and 1200 μg of DOC was obtained with a ratio hydrocortisone/DOC of 0.8/1. Potassium excretion was not enhanced when DOC and hydrocortisone were adminis-

TABLE 3. THE EFFECT OF DOC AND HYDROCORTISONE ADMINISTERED ALONE AND/OR SIMULTANEOUSLY ON ELECTROLYTE EXCRETION IN THE ADRENALECTOMIZED RAT

Material administered		Ratio hydrocortisone DOC	Total bladder content			% inhibitor of DOC effect on sodium	No. of animals
Hydrocortisone, μg	DOC, μg		Na, mg. ± SE	Na excretion, P*	K, mg. ± SE		
0	0		3.83 ± 0.31		5.55 ± 0.50		10
0	600		0.30 ± 0.08 (8)		9.32 ± 1.44 (1.68)		9
0	1200		0.26 ± 0.09 (7)		8.51 ± 0.21 (1.53)		5
500	0		4.26 ± 0.60 (111)		6.35 ± 0.76 (1.14)		9
1000	0		3.99 ± 0.78 (104)		6.39 ± 1.09 (115)		9
500	600	0.8/1	1.93 ± 0.33 (50)	<0.01	9.63 ± 1.12 (174)	46	8
1000	1200	0.8/1	2.98 ± 0.66 (78)	<0.01	7.12 ± 0.46 (128)	76	9

( ) % of the control value.
* Compared with treatment with DOC alone.

tered simultaneously as compared with the excretion observed in animals treated with DOC alone.

Effect of Progesterone on the Electrolyte Action of DOC

Tables 4 and 5 show the effect of DOC and progesterone when administered alone and/or simultaneously (at two different sites).

Typical sodium retention and potassium excretion were observed with dosages of DOC ranging from 6 to 200 μg. Progesterone, at 2000, 4000 and 6000 μg dosage levels did not induce any significant changes in electrolyte excretion as compared to values observed in control animals.

When 2000 μg of progesterone were given simultaneously with DOC, a ratio progesterone/DOC of 333/1 brought about 59% inhibition of DOC effect (P:0.05). With the same dosage of progesterone but a ratio of 80/1 no blocking was observed.

When 4000 μg of progesterone were used ratios of 666/1 and 160/1 produced 55 to 60 and 36% inhibition of DOC effect ($P \leq 0.02$). With ratios of 80/1; 40/1 and 20/1 the blocking effect was not statistically significant.

Increasing the dosage of progesterone to 6000 μg, ratios progesterone

TABLE 4. THE EFFECT OF DOC AND PROGESTERONE ADMINISTERED SEPARATELY AND/OR SIMULTANEOUSLY ON ELECTROLYTE EXCRETION IN THE ADRENALECTOMIZED RAT

Compound administered		Ratio progesterone DOC	Total bladder content			% inhibition of DOC effect on sodium	No. of animals
Progesterone μg	DOC, μg		Na, mg. ± SE	Na excretion, P*	K, mg. ± SE		
0	0		3.84 ± 0.48		4.61 ± 0.38		12
0	6		1.60 ± 0.20 (42)		6.20 ± 0.48 (134)		12
0	25		0.27 ± 0.04 (7)		6.49 ± 1.02 (141)		10
2000	0		3.66 ± 0.30 (95)		4.68 ± 0.36 (102)		12
4000	0		3.45 ± 0.21 (90)		5.29 ± 0.49 (115)		12
2000	6	333 1	2.93 ± 0.67 (76)	0.05	5.25 ± 0.43 (114)	59	10
2000	25	80 1	0.38 ± 0.09 (10)	>0.1 <0.5	5.99 ± 0.57 (130)	3	9
4000	6	666 1	2.95 ± 0.21 (77)	<0.01	4.13 ± 0.49 (90)	60	12
4000	25	160 1	1.60 ± 0.28 (42)	<0.01	6.91 ± 0.34 (150)	37	12
0	0		3.11 ± 0.81		6.78 ± 0.34		6
0	6		1.17 ± 0.35 (38)		9.82 ± 1.26 (145)		6
0	25		0.31 ± 0.12 (10)		8.50 ± 1.48 (125)		6
0	50		0.13 ± 0.03 (4)		8.24 ± 1.53 (122)		6
4000	0		3.48 ± 0.27 (112)		5.82 ± 0.99 (86)		5
4000	6	666 1	2.23 ± 0.19 (72)	0.02	6.30 ± 1.0 (93)	55	6
4000	25	160 1	1.32 ± 0.21 (42)	<0.01	7.64 ± 2.0 (113)	36	5
4000	50	80 1	0.71 ± 0.35 (23)	>0.1 <0.5	6.65 ± 0.57 (98)	20	6

( ) % of the control value.
* Compared with treatment with DOC alone.

/DOC of 120/1; 60/1 and 30/1 resulted in 40, 33 and 43% inhibition of DOC effect (<0.02).

Potassium excretion was not enhanced by the simultaneous administration of DOC and progesterone as compared to the excretion observed in rats treated with DOC alone. It should be noted that control excretion values for sodium and potassium as given in Tables 3 and 4 are higher than those presented in Tables 1 and 5. The difference observed falls within the variability of response obtained in this laboratory in control animals.

COMMENTS

The observations made in this study showed that, in the adrenalectomized rat, subjected to a sodium chloride load of 27 mg. hydrocortisone

TABLE 5. THE EFFECT OF DOC AND PROGESTERONE ADMINISTERED SEPARATELY AND/OR SIMULTANEOUSLY ON ELECTROLYTE EXCRETION IN THE ADRENALECTOMIZED RAT

Compound administered		Ratio progesterone DOC	Total bladder content			% inhibition of DOC effect on sodium	No. of animals
Progesterone, μg	DOC, μg		Na, mg. ± SE	Na, excretion P*	K, mg. ± SE		
0	0		2.90 ± 0.39		4.26 ± 0.22		11
0	50		0.50 ± 0.27 (17)		6.34 ± 0.54 (149)		8
0	100		0.19 ± 0.08 (7)		6.47 ± 1.04 (152)		12
0	200		0.08 ± 0.02 (3)		5.10 ± 0.62 (120)		6
4000	0		3.14 ± 0.42 (109)		4.26 ± 0.58		13
4000	50	80 1	1.20 ± 0.29 (41)	>0.05 <0.1	5.32 ± 0.43 (125)	29	8
4000	100	40 1	0.58 ± 0.16 (20)	>0.02 <0.05	5.75 ± 0.51 (135)	14	13
4000	200	20 1	0.32 ± 0.13 (11)	0.1	7.37 ± 1.87 (173)	9	4
0	0		3.90 ± 0.51		4.81 ± 0.49		9
0	50		0.53 ± 0.32 (14)		5.02 ± 0.79 (104)		10
0	100		0.48 ± 0.13 (12)		6.63 ± 0.47 (138)		10
0	200		0.35 ± 0.14 (9)		6.46 ± 0.50 (134)		10
6000	0		3.07 ± 0.32 (79)		4.52 ± 0.35 (94)		11
6000	50	120 1	1.86 ± 0.23 (48)	<0.01	4.66 ± 0.48 (97)	40	10
6000	100	60 1	1.61 ± 0.20 (41)	<0.01	6.35 ± 0.47 (132)	33	11
6000	200	30 1	1.88 ± 0.53 (48)	>0.01 <0.02	7.71 ± 0.83 (160)	43	10

( ) % of control value.
* Compared with treatment with DOC alone.

and progesterone are capable of increasing the excretion of sodium in the presence of DOC which, in itself, caused sodium retention under the same conditions.

This effect was not similar for all the dosages of hydrocortisone or progesterone tested. Hydrocortisone, at a dosage of 48 μg inhibited the sodium retaining activity of 6 μg of DOC. At a dosage of 100 and 200 μg, inhibition of DOC dosages ranging from 6 to 50 μg occurred. Increasing dosages of hydrocortisone to 500 and 1000 μg inhibition of DOC dosages ranging from 6 to 1200 μg took place.

Progesterone, at a dosage of 2000 μg, inhibited the sodium retaining activity of 6 μg of DOC. At a dosage of 4000 μg inhibition of 6 and 25 μg of DOC occurred. Increasing the dosage of progesterone to 6000 μg inhibition of DOC dosages ranging from 50 to 200 μg took place.

Uete and Venning (1) using a somewhat different assay system have shown that, in the adrenalectomized rat, hydrocortisone at dosages of 15, 100, 200 and 400 μg, inhibited the sodium retaining properties of 3 μg of DOC. The concomitant use of DOC and hydrocortisone brought about a greater potassium excretion than that obtained with DOC alone. Kagawa *et al.* (2), testing the effect of progesterone as a blocker of DOC in the adrenalectomized rat and utilizing urinary Na/K ratios as the end point, obtained 50% block of 12 μg of DOC with 1800 μg of progesterone.

The observations made during this study, as well as the ones reported by the authors mentioned above, failed to provide evidence as to the mechanism of action by which the blocking of DOC effect was brought about. This effect could be due to inhibition of tubular reabsorption of sodium, to increased glomerular filtration rate or to a combination of both.

A theoretical explanation could be offered if the following facts are considered: a) our own data and that of other workers (10–13) have demonstrated that hydrocortisone would induce increased sodium excretion when small dosages are used, and no effect or sodium retaining effect with larger dosages. The sodium retention was noted when a fixed time-interval of urine collection was employed and preceded sodium diuresis (12); b) the fact that increasing sodium chloride loads enhance this retention by influencing the amount of filtrated sodium as well as the amount of sodium reabsorbed by the tubules (14). These effects depend on the degree by which the effects of antidiuretic hormones are normalized and by the dose used (15–17).

If these facts are true, it would be possible to postulate that, small dosages of hydrocortisone may not influence tubular reabsorption of sodium, but may influence glomerular filtration as such. When combined with DOC, the small increase in filtrated sodium could not overcome the sodium reabsorption induced by the amount of DOC present, the net result would be sodium retention. When administered in combination with DOC large dosages of hydrocortisone would exert their sodium retaining

effect in addition to that induced by DOC. The increased sodium retention would bring about an immediate expansion of extracellular fluid which would result in an immediate increase in glomerular filtration rate. The sodium load reaching the tubules would be subjected to the combined effect of both steroids i.e.: maximum reabsorption but, due to the strong diuresis, the net effect would be sodium loss.

Progesterone has been demonstrated to exert sodium retaining activity in dogs (18). This action was suggested in rodents in which progesterone in large amounts supports the life of adrenalectomized animals (19–20). At the dosages used in this study, progesterone failed to elicit any influence on electrolyte excretion. Very large dosages of progesterone were necessary to induce sodium excretion in the presence of DOC. It would be possible to consider then, a different mechanism of action of progesterone compared to that postulated for hydrocortisone in that, progesterone may block the effect of DOC at the tubular level, without exerting an appreciable effect on sodium reabsorption or glomerular filtration rate.

It is evident that more experimental data as regards to the electrolyte action of hydrocortisone and progesterone would be necessary to substantiate these hypotheses.

The combination of DOC and hydrocortisone produced a greater excretion of potassium than that observed when DOC was administered alone. The increased potassium excretion could be due to a renal effect or to extrarenal effects such as an influence on intracellular potassium.

Keeping in mind the reservations made here concerned with mode of action, these data showed that 48 μg of hydrocortisone and 2000 μg of progesterone were required to produce approximately 50% block of 6 μg of DOC. By comparison of dosages hydrocortisone was demonstrated to possess, in the adrenalectomized rat, an activity 40 times greater than that elicited by progesterone in inducing sodium excretion in the presence of DOC.

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PROGESTERONE LEVELS IN THE RABBIT OVARIAN VEIN BLOOD THROUGHOUT PREGNANCY[1,2]

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ABSTRACT

The importance of progesterone in maintaining pregnancy in the ovariectomized rabbit is well established. A more thorough understanding of the progestational function of the ovary was attempted through the direct measurement of the values of progesterone in the ovarian venous blood at various stages of pregnancy.

Blood was extracted by a method similar to that of Butt and Zander. Qualitative identification and quantitation of progesterone was based on chromatography, U.V. absorption, isotope dilution and infra-red analysis.

The concentration of progesterone was found to rise gradually to reach a peak of 2.36 μg/ml. at mid-pregnancy; this was followed by a gradual decline in the second half of the pregnancy, with consistent low values of about 0.41 μg/ml. two days prior to parturition.

Similar determinations were made on peripheral blood. The curve was similar to that of the ovarian vein in its pattern but with lesser concentration. A ratio of about 1:5 was evident throughout.

IN 1929 Allen and Corner (1) began a series of experiments on pregnant rabbits which proved that castration in very early pregnancy prevents early development and implantation and that the administration of corpus luteum extracts maintains pregnancy to term in rabbits castrated 24 hours after mating. It is also well known that castration during pregnancy is followed by abortion or premature delivery. In 1958 Wu and Allen (2) were able to maintain pregnancy in rabbits castrated 24 hours after mating with either progesterone or 17-alpha-hydroxyprogesterone caproate providing a suitable dose was given. These experiments point out the importance of the ovary in the rabbit for the maintenance of pregnancy and indicate that progesterone is probably the essential hormone produced by the ovary during pregnancy. Also, there seems little liklihood that there

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is a significant extragonadal source of progesterone in the pregnant rabbit.

The aforementioned experiments do not, of course, prove beyond doubt that the actual hormone produced by the ovary of the pregnant rabbit is progesterone. The evidence indicates only that progesterone is an adequate progestational agent for the rabbit. To be more certain that progesterone is the progestational agent of the rabbit it is necessary to either isolate progesterone from the ovaries of the rabbit, or preferably, to measure the amount of progesterone in the blood of the ovarian veins. The present experiments were designed primarily to find out whether or not progesterone as such is produced by the rabbit ovary and secondarily to ascertain the fluctuation in the level of production of progesterone which may occur as pregnancy progresses.

MATERIALS AND METHODS

For all the studies white New Zealand rabbits, weighing from eight to ten pounds, were used. Pregnancy was verified at laparotomy by observing the number of fetuses present or by recovering the blastocysts in the cases studied prior to implantation. The number of corpora lutea was also recorded. All operations were performed under nembutal anesthesia, supplemented with ether. The ovarian vein of the rabbit was isolated and all its non-ovarian tributaries were ligated. Ten mg. of heparin were injected intravenously in the marginal ear vein. A small polyethylene catheter was then inserted in the ovarin vein and blood allowed to drip into a flask. Sometimes both ovarian veins were tapped simultaneously. Care was taken to cover the abdominal viscera and to keep the animal warm while the blood was dripping. A 50 cc. blood sample was usually collected in a period of 45–75 minutes. A few samples were obtained from the aorta and heart for measurements on peripheral blood.

The extraction of the progesterone from the whole heparinized blood was carried out as follows:

a) A tracer amount of 4-C^{14} progesterone of known specific activity was added to the blood which was extracted immediately with 10 volumes of ether-ethanol 1:3, centrifuged and the supernatant fluid evaporated to dryness. The residue was taken up in ethyl acetate and water and then extracted with equal volumes of ethyl acetate. The water was discarded and the ethyl acetate evaporated to dryness and the residue dissolved in 70% methanol. This was then chilled at $-15°$ C for 24 hours and centrifuged to separate the lipids. The 70% methanol was diluted with two volumes of water and extracted with equal volumes of light petroleum ether (B.P. $35°-45°$ C) yielding a progesterone concentrate suitable for paper chromatographic purification (3, 4). Number two Whatman filter paper, pre-washed at least 48 hours with absolute methanol, was used. The Skelly C-formamide system was employed. After development of the chromatogram, the progesterone zone was located by both U.V. light absorption and by the radioactivity of the tracer progesterone. The progesterone spot was cut and eluted; a corresponding blank piece of paper was also eluted. Both the progesterone and the blank eluates were transferred to the spectrophotometer cells and the U.V. absorption curve was measured.

The validity of the quantitative measurements of progesterone in the sample is dependent upon the "purity" of the absorption curves obtained. The "purity" of the absorption curve of the sample was compared with curves obtained with comparable amounts of pure progesterone. To obtain reference curves, standard solutions of progesterone containing amounts varying from 1.25 μg/ml. to 20 μg/ml. were prepared

and the absorption spectra determined. From these curves two standard K values were obtained.

$$K_1 = \frac{\text{Density at } 240}{\text{Density at } 230}$$

$$K_2 = \frac{\text{Density at } 240}{\text{Density at } 250}$$

These two constants actually serve as measures of the ratio of the density at the point of maximum absorption (240 mμ) to the absorption at two fixed points on either side of the maximum (230 mμ and 250 mμ).

The ultraviolet absorption curves of the various samples were considered for quantitation only when both their K_1 and K_2 values fell within the range of the values for pure progesterone of similar amount. When the curves of the sample did not qualify for quantitation, the sample was subjected to a second chromatographic separation. The actual progesterone content of the original blood sample was finally calculated from the percentage of the original radioactivity found to be present in the final fraction on which the ultraviolet absorption spectrum was obtained. The yields actually varied from 55–70%.

The question may probably arise as to whether or not the material finally determined as progesterone was really progesterone. The supposition is, of course, that the compound is progesterone. The method of preparation of the extract is one known to yield progesterone; the mobility of the extracted compound in the Skelly C-formamide system is the same as the mobility of progesterone in this system and identical with that of the admixed authentic 4-C^{14} progesterone. Furthermore, the U.V. absorption spectrum is the same. Finally, the identification of the extracted compound as progesterone was established by infra-red absorption. To obtain the infra-red absorption spectrums, the samples on which the ultraviolet absorption was determined were pooled and further purified by absorption and elution from an alumina column. A 20 microgram aliquot was used for the determination of the infra-red spectrum. There was good agreement between the extracted sample and authentic progesterone (Fig. 1).

RESULTS

The values obtained for the concentration of progesterone in ovarian venous blood are given in Figure 2. A level of 0.4 μg/ml. on 2nd day after mating rose gradually to 1.51 on day 10, to reach a maximum of 2.36 on day 16. From there the level dropped gradually to 1.95 on day 20, 1.07 on day 29, followed by a consistent drop to 0.62 on day 30, 0.44 on day 31 and 0.41 during parturition. No progesterone was detected four days post partum.

A smaller number of pregnant rabbits were used for the determination of progesterone in the peripheral blood. The values obtained were much lower than the values obtained from the venous blood. The maximum value was still found at the same stage of gestation (16th day) as the maximum found in the ovarian venous blood. A level of 0.14 μg. was present on the 10th day, 0.43 μg. on the 16th day and 0.06 μg. on the 23rd day (Fig. 2).

DISCUSSION

The levels of progesterone in the peripheral blood of the pregnant rabbit have been previously studied by Zarrow and Neher (5) using the Hooker

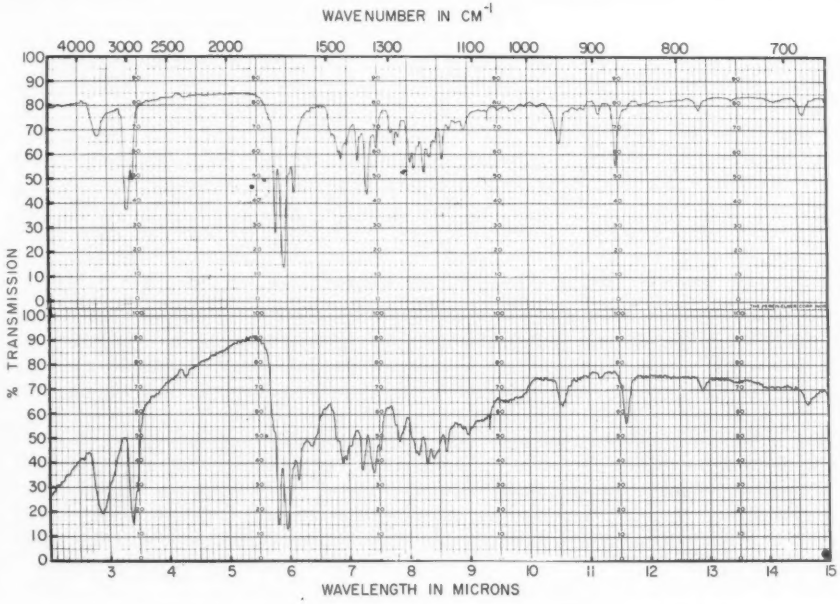

FIG. 1. Infra-red absorption spectra of authentic progesterone (upper curve) and progesterone extracted from ovarian-vein blood.

and Forbes method of bioassay. Our results are in marked contrast, not only in the magnitude of the values they obtained by the bioassay, but also in the pattern of their curve of progesterone during pregnancy; they illustrated a curve with values continuing to rise up to 30 minutes post

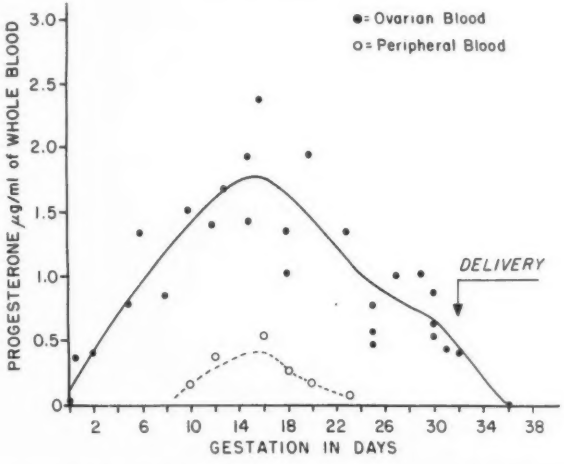

FIG. 2. Progesterone levels in whole blood at various stages of pregnancy in the rabbit.

partum. Our results from the ovarian blood, on the other hand, show a curve that reached its peak at midpregnancy, declining gradually thereafter with a consistent drop two to three days prior to delivery. In fact, our peripheral blood values seem to follow the same pattern as the ovarian only in far smaller concentration.

Edgar and Ronaldson (6) measuring levels of progesterone in the ovarian vein and uterine vein of ewes report that no progesterone could be detected two weeks prior to parturition. Short (7) measuring peripheral blood levels of progesterone in the pregnant cow shows a consistent drop of the level of progesterone before parturition. Kimura and Cornwell (8) found by bioassay that the progesterone content of sows ovaries rises rapidly during the first two weeks of gestation and then remains at a fairly constant level until near term. Assays of ovaries shortly prior to term showed no progesterone. Forbes and Hooker (9) showed similar results in their determination of the progesterone concentration in the pregnant mouse. Our results from ovarian venous blood are, therefore, in accord with the above observations.

In the pregnant rabbit it is well established that castration leads to abortion; that pregnancy in the castrated animal can be maintained with exogenous progesterone; that administration of progesterone at term will delay parturition (10). To this we add another fact, that the production of progesterone consistently drops as term approaches. It seems logical then to conclude that the high values of progestins reported by Zarrow and Neher before and during parturition represent compounds other than progesterone that give a positive result in the Hooker Forbes bioassay, but do not exhibit any inhibitory effect on the act of parturition in the rabbit.

There are several biological changes which occur in the rabbit in the latter part of pregnancy that are of interest in the light of the decreasing levels of progesterone as term approaches. First, the mammary glands begin to secrete milk several days prior to delivery, and second, the rabbit usually builds a nest of fur about two days before delivery. At least, in the case of parturition delayed by the administration of progesterone, nest building is likewise usually postponed. The decreasing levels are in keeping too with the observations by Heckel and Allen that the sudden withdrawal of progesterone, after treatment for 25 days with estrogen and progesterone in castrated non-pregnant rabbits, results promptly in nest building and lactation. Likewise, the escape of the myometrium from progesterone dominance in the last two or three days of pregnancy points to relative progesterone deficiency. It appears then that the decreasing levels of progesterone as the time for parturition approaches are related in some significant way to the biological changes which ultimately result in birth of the fetuses.

The actual levels of progesterone observed during pregnancy are in

keeping with the known amounts of progesterone required to maintain pregnancy in the castrated, pregnant rabbit. Heckel and Allen found that 4.0 mg. daily was adequate to maintain pregnancy when castration was done on the 12th day, whereas 2.0 mg. was inadequate. Wu and Allen also noted that 2.0 mg. of 17-alpha-hydroxyprogesterone caproate was sufficient to maintain pregnancy. While we have no really accurate way of measuring the total daily output from the ovaries, a rough approximation is possible. Usually 40 ml. of whole blood was obtained from one ovarian vein in about one hour. Assuming a value of 1.5 μg per ml. per hour from each ovarian vein, we obtain a value of 2.88 mg. for the daily output at the mid-point of pregnancy. Similarly, by using a value of 0.4 μg per ml. at the end of pregnancy for the calculation, the daily output is only .77 mg. per day. These calculations certainly indicate that the amount of progesterone elaborated by the ovary is in the same order of magnitude as the amount required when given subcutaneously in oil to maintain pregnancy in the castrated rabbit.

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IODINE CONTAINING SUBCELLULAR PARTICLES IN THYROID TISSUE[1]

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ABSTRACT

Iodine-containing subcellular particles in homogenates of a transplantable thyroid tumor in rats have been shown to be heterogeneous with respect to density and size. Three more or less distinct groups of such particles have been isolated on the basis of particle size. Two of these would be isolated with the "mitochondrial" fraction and one with the "microsomal" fraction by classical techniques.

Similar groups of iodine containing particles have been found in non-tumorous thyroid tissue from various animals. The distribution of iodine in the particles of hog thyroid is similar to the distribution in the tumor particles.

The two groups of smaller iodine containing particles in hog thyroid tissue correspond closely with particles from this tissue which produce a particulate iodoprotein in a cell-free system.

No role in hormone biosynthesis can yet be assigned to these particles.

THE endoplasmic reticulum of thyroid cells as observed by electron microscopy changes with secretory activity of these cells (1, 2, 3). It is not clear whether these observations imply that iodinated materials are associated with this structure. Recent studies, however, have shown that a small amount of iodine is found in subcellular particles. Rerabek (4) found that 2.7% of the thyroxine and 4.7% of the diiodotyrosine of hog thyroid tissue were in the nuclear fraction. Negligible amounts of these amino acids were found in the large and small granule fractions (Rerabek's Chondriome I and II).

Taurog *et al.* (5) assayed nuclei and a mixture of mitochondria and microsomes from sheep thyroid for total iodine content. Their results show that the nuclear fraction contains 0.35–3.0% of the total thyroid iodine while the combined mitochondrial-microsomal fraction contains 3.3–4.7% of the total iodine. The remainder was found in the soluble fraction.

Recently I^{131} has been used to indicate the iodine content of subcellular particle fractions. Robbins *et al.* (6) showed that all subcellular particles combined concentrate only about 5% of the I^{131} in normal thyroid glands

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labeled *in vivo*. Similar results were obtained with *in vitro* labeled thyroid slices. DeGroot (7) found a similar amount of I^{131} incorporated into subcellular particles.

The distribution of I^{131} in soluble and particulate portions of the gland is different in abnormal thyroid tissue than in normal tissue. Robbins (6) *et al.* found up to 37% of the total I^{131} concentrated by human thyroid neoplasms to be in the particulate fractions. A transplantable rat thyroid tumor (8) concentrated up to 50% of the total iodine incorporated by the tumor in the subcellular particles. Early experiments suggested that most of the particulate I^{131} in this tumor was associated with the nuclear fraction. Nuclear fractions were prepared and most of the "nuclear" I^{131} was solubilized from the particles as an iodoprotein. Preliminary reports on some of the physical properties of this protein demonstrated that it is not thyroglobulin (9, 10).

DeGroot (11) also found that particulate iodine is increased in human thyroid carcinomas, and he described two cases of congenital goiter, in one of which particulate iodine was decreased while in the other it was increased. DeGroot and Carvalho (12) later solubilized an iodoprotein from the mitochondrial fractions of both sheep and human thyroid glands using deoxycholate, and found that this protein is distinct from thyroglobulin in sedimentation constant and immunologic behavior.

Other studies have shown that enzymes capable of catalyzing iodination reactions are also found in particulate fractions of thyroid cells. Weiss (13) showed that cell-free suspensions of thyroid tissue particles can organify iodine if supplemented with tyrosine and copper. The nuclear and mitochondrial fractions were found to be the most active. More recently Taurog and his group (5) have found that the cell-free particles incorporate I^{131} without supplementation by copper and tyrosine. Flavine co-factors in the presence of light and certain quinones (14) increased incorporation by the particles, which are most active at pH 9–10. Anaerobiosis is inhibitory to this system (15). Subcellular fractions were isolated in Krebs-Ringer bicarbonate solution using conventional centrifugal forces, and it was found that the nuclear fraction has little iodinating activity while the mitochondrial and microsomal fractions are about equally active.

Tong and Chaikoff (16) have studied the rate of diiodotyrosine formation in the soluble and particulate portions of *in vitro* labeled thyroid slices and *in vitro* labeled thyroid homogenate. In both systems the MIT/DIT ratio of the particles is 10–15 after 15 minutes of incubation with I^{131} and falls to about 4 after 2 hours. The soluble iodoprotein shows a ratio of 3–5 initially which also decreases with time. These experiments suggest that the iodination of particles observed in cell-free systems proceeds similarly to that observed in intact cells.

Morphological and biochemical studies thus suggest that subcellular particles have a role in the iodine metabolism of the thyroid cell. The fol-

lowing experiments are concerned with a further study of the particles from a rat thyroid tumor. Similar iodine containing particles are described in normal thyroid tissue, and the possibility that these iodine containing particles may have some role in iodination is investigated.

MATERIALS AND METHODS

Thyroid Tissue—One of the functional rat thyroid tumors developed by Dr. S. H. Wollman (17) of the National Cancer Institute (Wollman's line 1–8) was used for these experiments because it concentrates an unusually large amount of particulate iodine. This is the same tumor that was used in earlier studies on particulate iodoproteins (8, 9). The tumors were used two to three months following bilateral subcutaneous transplantation, at which time they weighed 3–7 g. each. Other sources of thyroid tissue are described in the section on experiments.

Labeling—For *in vivo* labeling of tumor bearing rats, 500 μc of I^{131} was injected intraperitoneally into each animal. Calculations of tumor radiation dosage (8) showed that the total irradiation was less than that which would cause normal thyroids to discharge thyroglobulin into the blood. 48–60 hours were allowed to elapse between injection and sacrifice of the animals to provide maximum labeling of the subcellular particles. The animals were then etherized and exsanguinated, and the tumors were dissected from their subcutaneous locations and trimmed of extraneous tissue.

Measurement of I^{131}—Radioactivity was assayed with well-type scintillation counters with sodium iodide crystals. The probable error in counting was below 3–5%.

Homogenization—The tissues were sliced with a razor blade and homogenized using a Potter-Elvehjem motor-driven glass homogenizer immersed in an ice-water bath (0° C). The homogenate was strained through coarse mesh cotton gauze.

Centrifugation—Two instruments were used for centrifugation: the International Refrigerated Centrifuge and the Spinco Model L refrigerated ultracentrifuge. Centrifugation was done at 0–20° C. Centrifugation techniques are described in the next section.

EXPERIMENTS AND RESULTS

Autoradiography

Early attempts to fractionate I^{131} labeled rat tumor tissue in 0.88 M sucrose by the technique of Hogeboom *et al.* (18), suggested that the nuclei contained most of the particulate radioiodine although the two other particulate fractions also contained variable amounts of iodine (8). In an attempt to provide additional evidence localizing the particulate radioactivity to the nuclear fraction, autoradiography was performed with labeled tumor nuclear fractions by Dr. L. Schroeder of the National Cancer Institute using Kodak Fine Grain Autoradiographic Stripping Film (AR 10). The homogenate was applied to a glass slide as a smear and the film applied on top of this. The exposure time was 2 weeks. As shown in Figure 1, the oxidized silver grains in the film are associated with some aggregated material which cannot be seen in detail by light microscopy. The nuclei, stained with Giemsa stain, are not associated with grains, and the radioactivity originally believed to be in the nuclei must have been in the contaminants of the nuclear fraction.

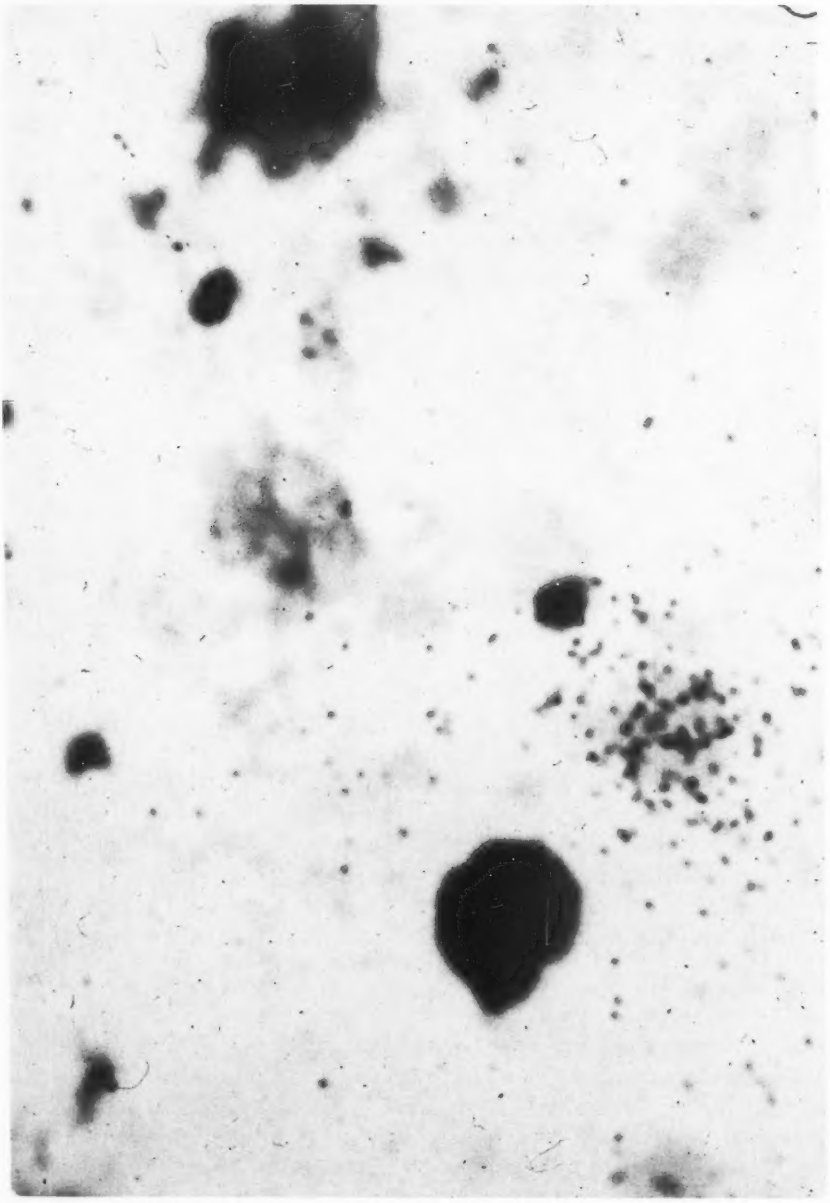

FIG. 1. Autoradiograph of the homogenate from the rat tumor (approximately 6200×). Two nuclei are shown in this figure, however, neither of them is associated with film granules which would indicate radioactivity. Instead the radioactivity is associated with aggregated non-nuclear particles. The right hand portion of the illustration shows these radioactive particles.

Tissue Fractionation Experiments

I. Classical Techniques of Differential Centrifugation

The distribution of iodine in the classical subcellular fractions was reinvestigated using several methods of differential centrifugation. In all cases the tumors were homogenized in ten times their weight of the appropriate solution, and the homogenates were filtered through gauze before use. The forces described are the average (midtube) forces applied.

Method A. This method of Hogeboom (19) involves homogenizing tissue in a 0.25 M sucrose solution, precipitating a nuclear fraction by centrifugation for 10 minutes at 600 g, precipitating a mitochondrial pellet from the resulting supernatant at 24,000 g for 10 minutes, and from that supernatant, precipitating a microsomal fraction by centrifugation at 54,000 g for one hour. This method was tried with and without the addition of 1.8×10^{-4} M $CaCl_2$ to the sucrose solution.

Method B. The technique of Hogeboom *et al.* (18) using 0.88 M sucrose was also employed. This technique includes the preparation of a nuclear fraction in a two phase system. The homogenate in 0.88 M sucrose is lighter than the clear 0.88 M sucrose solution, and is, therefore, easily layered over twice its volume of clear sucrose solution. Centrifugation at 600 g for 10 minutes precipitated the nucler fraction. The supernatant from the nuclear precipitation was centrifuged for 20 minutes at 24,000 g to sediment mitochondria, and the supernatant resulting from that centrifugation was centrifuged at 41,000 g for two hours to precipitate microsomes. Preparations of fractions were done with and without the addition of $CaCl_2$ to the sucrose solution.

In these tissue fractionation experiments, the nuclear fractions were prepared in the refrigerated International centrifuge using a horizontal (#269 or 284) rotor. The other fractions were prepared in the Spinco Model L centrifuge using an angle (#30 or #40) rotor.

The results of these experiments are at some variance with the originally published results (8) as shown in Table 1. Most striking about these results

TABLE 1

Technique of cellular fractionation used	Percentage of total tumor I^{131} present in:			
	Nuclear fraction	Mitochondrial fraction	Microsomal fraction	Soluble fraction
Method A—0.25 M sucrose				
1. With calcium	9	18	18	55
	17	22	21	40
2. Without calcium	1	7	7	85
	2	10	32	56
Method B—0.88 M sucrose				
1. With calcium	21	17	6	56
	24	31	8	37
2. Without calcium	13	13	13	61
	12	29	15	44

is the variation with the method employed, both in the amount of particulate iodine contained in different tumors, and in the distribution of this iodine among the various subcellular fractions. The addition of calcium to the fractionating medium increases the amount of iodine appearing in the large particle fractions. Other investigators (20) have described clumping of small granules in the presence of calcium. These results show variable amounts of radioactivity incorporated by the nuclear fraction, although the autoradiographic results make it evident that this "nuclear" iodine is present in the contaminants of the fraction.

II. Sucrose Gradient Centrifugation

The appearance of substantial percentages of iodoprotein in both the mitochondrial and microsomal fractions as isolated by several techniques indicated that the iodinated particles do not fall into any single narrow range of size. Sucrose gradient centrifugation has been used to prepare subcellular constituents which are not separable by classical fractionation techniques (21, 22, 23, 24). It has also been used as an analytical tool to determine sizes of particles containing various enzymes or compounds (25, 26). In this study sucrose gradient centrifugation was used to determine whether the appearance of I^{131} in both the mitochondrial and microsomal fractions indicated particles of a wide range of density or whether there were several discrete classes of particles. Numerous gradients which had been devised for various purposes by other workers were tried, and a slight modification of a gradient used by Kuff *et al.* (27) was finally selected This gradient is prepared by layering 0.5 ml. of each of the following sucrose solutions in the order indicated in the small plastic centrifuge tubes manufactured for the SW 39 swinging bucket rotor of the Spinco Model L centrifuge: 2.5 M sucrose at the bottom, 2.2 M, 1.8 M, 1.6 M, 1.4 M, 1.2 M, and 0.65 M. Over the top sucrose layer, 0.5 ml. of tumor particles suspended in 0.25 M sucrose was layered. The tumor particles used for these experiments and others described below were prepared by centrifuging the tissue homogenate in a two phase system (0.88 M sucrose) for 70 minutes at about 100,000 g in one of the angle heads (#30 or #40) of the Spinco Model L centrifuge.

Equilibrium of the system was obtained by centrifugation at 150,000 g for 18 hours in the SW 39 rotor. At the termination of centrifugation the contents of the centrifuge tubes were removed in small fractions through a hole punched in the bottom of each tube. Prior to making the hole, a cork with a hypodermic needle through it was placed in the mouth of the tube, and a syringe full of air was attached to the needle so that the expression of the contents of the tube could be controlled. The fractions were counted and their volumes were determined.

The distribution of I^{131}-containing particles in a representative gradient experiment is shown in Figure 2. Two peaks of activity are evident which

indicate iodine containing particles suspended at different levels in the tube. The larger of the peaks contains 50% of the total activity added to the gradient. Experiments show that the original sucrose gradient remains relatively unaltered during centrifugation. The particles in the large peak correspond with layers of 1.4 and 1.6 M sucrose, indicating that these particles have come into equilibrium with solutions of approximate density 1.19 to 1.21,[3] and that the particles themselves are of this density. The smaller peak represents 17% of the particulate I^{131} added to the gradient. This peak contains particles at equilibrium with layers containing 0.65 to 1.2 M sucrose. These particles therefore, are of density 1.09 to 1.16. There is another concentration of iodinated particles which is not evident from the figure. These are particles which appear in a small pellet at the bottom of the gradient tube following centrifugation. Puncturing the bottom of

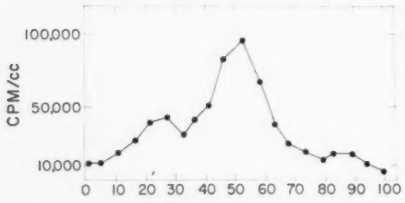

FIG. 2. The distribution of I^{131} containing particles in a sucrose gradient after centrifugation. Ordinate values show counting rates (counts/minute/cc.) of fractions removed from a sucrose gradient after centrifugation. Abscissa values show the depth in the gradient from which the fractions were removed as the percentage of the distance from the meniscus to the bottom of the tube. This experiment is representative of several which were performed.

the tube to remove the rest of its contents caused no visible disturbance to this pellet. The particles composing this pellet have sedimented through a layer of 2.5 M sucrose, and therefore have a density greater than 1.32. They also account for 17% of the iodinated material in the illustrated gradient.

The appearance of three groups of iodinated particles was constant in all gradient experiments performed on tumor particles. The location of these groups of particles in the gradient was also quite constant, although the percentages of total radioiodine contained in each group of particles was somewhat variable. In some cases the least dense particles contained a larger percentage of the total particulate radioiodine than the particles of intermediate density.

Since these gradient experiments employed isopycnic centrifugation which separates particles on the basis of density alone, nothing can be said about the sizes of the particles from these results (21). The experiments show, however, that there are three groups of iodine containing particles separable on the basis of their densities.

[3] Density values are those given by DeDuve *et al.* (28).

III. A Technique for Surveying Homogenates for Particulate Distribution of I^{131}

For further study of the iodine containing particles, a modified two phase technique was devised which will be described in more detail in another publication (29). In this technique, 0.5 ml. aliquots of tumor particles suspended in 0.88 M sucrose solution were layered over 3.0 ml. of clear 0.92 M sucrose solution. A series of these preparations was centrifuged at a wide range of forces for varying periods of time. After centrifugation the resulting supernatants and precipitates were counted for I^{131} content. The increment in the percentage of I^{131} precipitated by each successive force

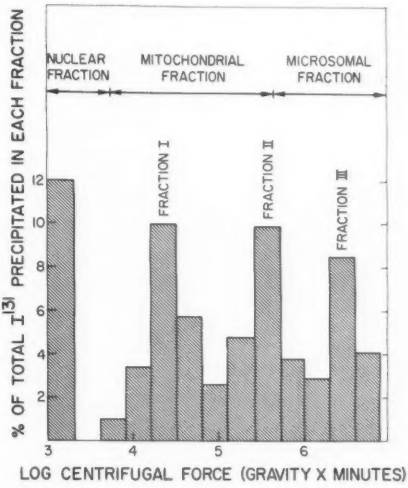

FIG. 3. The distribution of I^{131} in fractions from the rat tumor. In this figure the percentage of the total I^{131} in the tumor particulate which is precipitated in each fraction is plotted against the centrifugal forces defining the fraction. The fractions containing radioactive particles, designated Fractions I, II, and III appear as three peaks. Another fraction containing much I^{131} is precipitated by the lowest centrifugal force employed. The top of the figure shows the relationship of the classical subcellular fractions to the iodine containing fractions and to the forces used in this experiment.

was plotted against the integral force used. It has been shown (30) that the integral centrifugal force may be approximated by multiplying the force used by the time during which it was applied, and the product is given in gravity-minute units.

By this technique a large number of subcellular fractions were prepared from the tumor particles, and studied for I^{131} content. The results of the survey are shown in Figure 3. The iodine containing particles sedimented in three distinct groups, called Fractions I, II and III. This suggests that iodine is not randomly distributed among particles of all sizes. Fractions I and II would ordinarily both be isolated with mitochondria although these fractions are distinctly separate from each other. Fraction III would be described as microsomal, although it is precipitated by a much narrower range of conditions than is used to precipitate the whole microsomal fraction by classical techniques. There is, in addition to Fractions I, II and III, a group of iodine containing particles which is precipitated by the

lowest force employed, and which would ordinarily be considered part of the nuclear fraction. Because of the autoradiographic experiments, however, we believe that this represents iodine contained in incompletely homogenized cells and in aggregations of smaller particles and cells.

The sizes of the particles in each fraction were calculated using the following expression, which can be derived from the formulas given by DeDuve and Berthet (30):

$$d^2 = \frac{63\eta(\log_{10}R_{max} - \log_{10}R_{min})}{(\sigma - \rho)(RPM)^2 T}$$

where d = particle diameter (cm.), η = viscosity of the suspending medium (poises), T = time for sedimentation (min.), σ = particle density (gm./cc.), ρ = density of the suspending medium (gm./cc.), R_{max} = distance from the axis of rotation to the bottom of the centrifuge tube (cm.), R_{min} = distance from the axis of rotation to the surface of the suspension in the tube (cm.), and RPM = speed of centrifugation (revolutions per min.).[4] For this calculation it was assumed that the particles are spherical and that the particle density is 1.22. This figure for density is only approximate and is derived from the average found in the density gradient experiment. It has not yet been possible to correlate the groups of particles as they appear in the density gradient with the three fractions found by the survey technique. Furthermore it is not known whether these particles are subject to change in density due to osmotic forces. If they are, then the density used in the calculation (for sedimentation in 0.92 M sucrose) is too high. Consequently, the values calculated provide only a rough approximation of the actual particle sizes.

With these assumptions, the particles of Fraction I have a calculated range of diameter of 1.6–2.1 microns. Those of Fraction II are 0.37–0.53 microns in diameter, and those of Fraction III are 0.13–0.19 microns in diameter. The group of I^{131} containing particles which sediments with the smallest centrifugal force employed (2,000 g-min) have diameters greater than 5.8 microns. This calculation supports the contention that this group of particles contains aggregated material and incompletely homogenized cells in addition to nuclei.

Preparation and Photomicrography of the Tumor Iodine Containing Particles

The survey of subcellular particles in the tumor tissue for iodine indicates centrifugal conditions which can be used to isolate the iodine containing particles with more precision than can be done with classical tissue fractionation techniques. Fraction I may be isolated by centrifuging the two phase preparation described for the particle survey technique for 20

[4] Values for density and viscosity of the suspending medium are given by DeDuve *et al.* (28).

minutes at 800 g in the International refrigerated centrifuge using a horizontal (#260) head. The supernatant from this first centrifugation is divided and layered over 3.0 ml. of 0.92 M sucrose in each of three small centrifuge tubes. These tubes are centrifuged for 10 minutes at 3200 g in the Spinco horizontal (SW 39) rotor. The small pellets resulting from this centifugation contain the desired particles. A similar procedure was followed to isolate the particles in Fraction II except that the first centrifugation was for 10 minutes at 25,600 g and the second was for 10 minutes at 51,200 g. To prepare the third group of particles the first centrifugation was for 12 minutes at 170,000 g and the second was for 24 minutes at 170,000 g. These forces indicate the forces applied at the bottoms of the tubes (i.e. the maximum forces).

These preparative techniques were used to prepare iodinated particles for microscopy. The small size of the particles precluded useful observations on them by light microscopy. For electron microscopy the unfixed particles of Fraction I were suspended in water, mounted, and shadow-casted with palladium. All electron microscopy was kindly done by Dr. Louis W. Labaw of the National Institute of Arthritis and Metabolic Diseases. Fraction I contained chiefly spheres 0.4–1.0 microns in diameter which are shown in Figure 4. Successful photomicrographs have not yet been made of the particles of Fraction II and III because of clumping. It is, of course, unknown whether the particles illustrated are the ones containing iodine or whether they are some of the non-iodinated particles which also undoubtedly occur in the fractions. The disagreement in the sizes of the particles in Fraction I as determined by electron microscopy and by calculation allows no conclusion since changes may have occurred during preparation for electron microscopy.

Nitrogen and I^{131}/Nitrogen Ratios in the Tumor Particles

The total nitrogen in each fraction isolated by the survey technique was determined by the micro-Kjeldahl technique.[5] Prior to nitrogen assay these same fractions were counted for I^{131} so that iodine/nitrogen ratios of the fractions could be determined. The distribution of nitrogen in the tumor plotted in the manner already described is shown in Figure 5. Nitrogen is quite evenly distributed throughout the fractions of the tumor with two exceptions. The lowest force employed precipitates about one-third of the total particulate nitrogen in the tumor, and there is a concentration of nitrogen corresponding with Fraction I of the iodine containing particles.

The I^{131}/nitrogen ratios were calculated as the percentage of total particulate I^{131} in each fraction divided by the percentage of total particulate nitrogen. The ratios, which are shown in Figure 6, emphasize the dissimilarity of I^{131} and nitrogen distribution. This dissimilarity suggests that the

[5] These analyses were performed in the analytical laboratory of the National Institute of Arthritis and Metabolic Diseases.

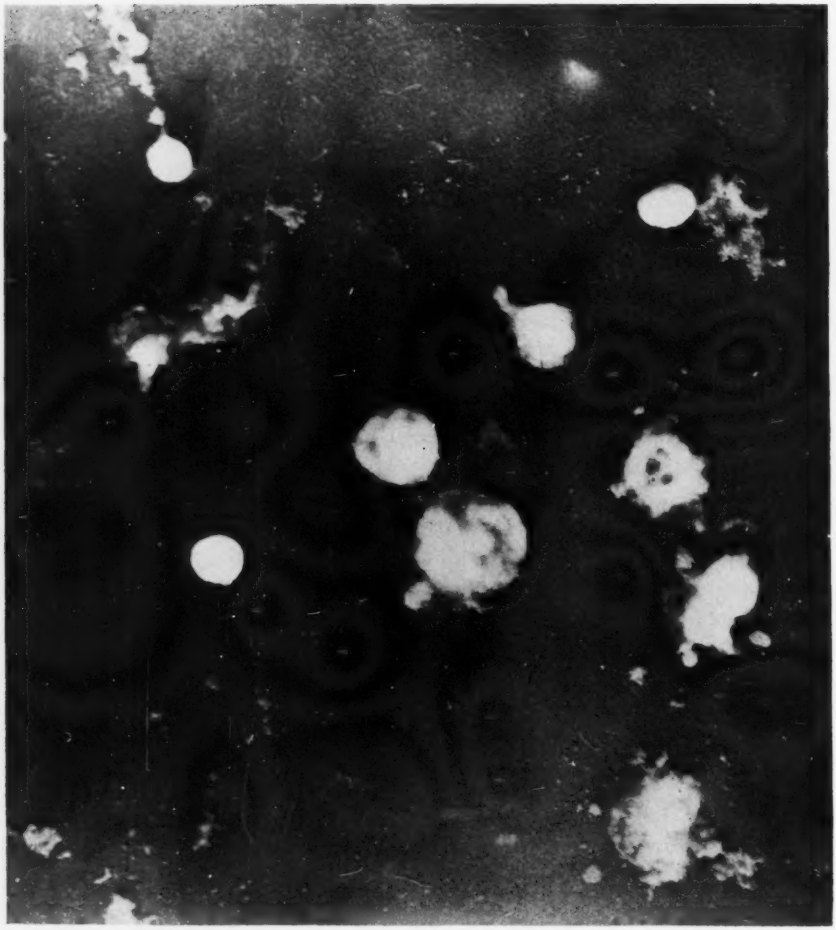

FIG. 4. Electron photomicrograph of particles in Fraction I (16000×). The particles were dried on and shadow-casted with palladium.

appearance of iodine in subcellular particles of the tumor tissue is not the result of non-specific adsorption of soluble iodoproteins onto particles. In general, the fractions showing a concentration of I^{131} show high I^{131}/nitrogen ratios. The extremely large ratios found for the smaller iodine containing fractions are the result of the minute amounts of nitrogen associated with these small particles.

Phosphorous in Tumor Particles

Phosphorous was determined in one series of fractions by the phosphomolybdic acid method[3] in the fractions so that the distribution of phos-

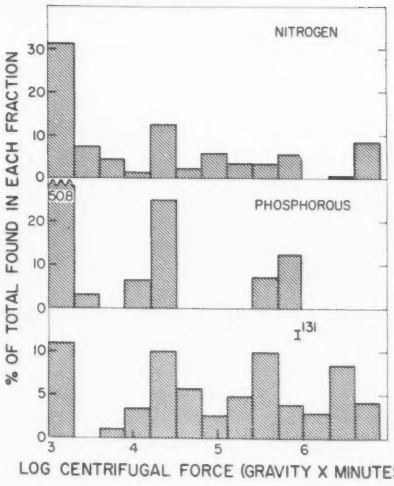

FIG. 5. Survey of rat tumor tissue for nitrogen, phosphorus and I^{131}. The distribution of particulate nitrogen and phosphorus with respect to particle size is compared with the distribution of I^{131} with the results plotted as in Figure 3.

phorous containing compounds in subcellular particles might be compared with that of I^{131}. As shown in Figure 5, there are three concentrations of phosphorous in the tumor tissue, one corresponding with the largest particles of the nuclear fraction as isolated by classical techniques, one corresponding with the mitochondrial fraction and with Fraction I of the iodine containing particles, and one corresponding with Fraction II of the iodine containing particles. Although there are many phosphorous containing compounds in particles, these results suggest the possibility that some of the iodine containing particles may also contain abundant nucleic acid which may have some role in the synthesis of the particulate iodoproteins.

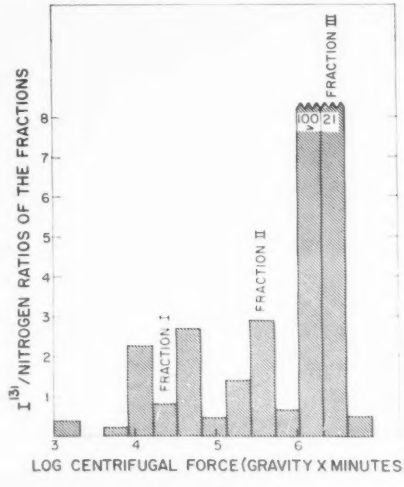

FIG. 6. I^{131}/Nitrogen ratios of the tumor fractions. The results shown in Figure 5 are plotted as I^{131}/nitrogen ratios. The ratios emphasize the dissimilarity of I^{131} and nitrogen distribution among the fractions.

I^{131} Distribution in Particles of Normal Thyroid Tissue

Normal thyroid tissue was also surveyed for the distribution of iodine in subcellular particles. Normal rabbit thyroid was labeled *in vivo* by injecting 150 μc of I^{131} intraperitoneally and sacrificing the animal 48 hours later. About 10% of the total thyroid iodine was found in the particles. Figure 7 shows the distribution of I^{131} in the particle fractions. A large amount of iodine sedimented with the lowest force employed, which may well have been the result of inadequate homogenization of the gland. A group of particles sedimenting under conditions comparable with Fraction I of the tumor homogenate was found, and there is a concentration of

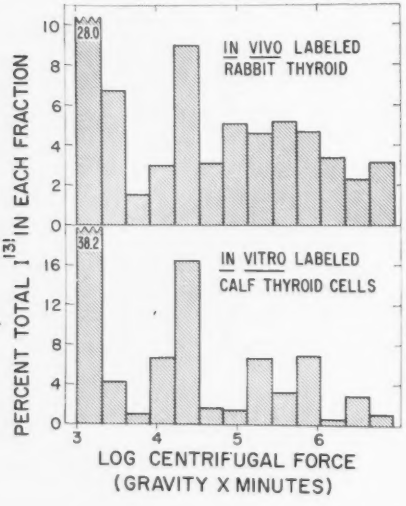

FIG. 7. A Survey of normal thyroid tissue for particulate I^{131}. The distribution of I^{131} in the fractions of these normal thyroid preparations is to be compared with that in a third normal preparation shown in Figure 8 and with that in the tumor shown in Figure 3. With the exception of one fraction corresponding to Fraction I in the tumor, none of the fractions containing concentrations of I^{131} in these two preparations correspond exactly with the iodine containing fractions in the tumor tissue.

iodine contained in particles smaller than those of Fraction I which have a wide range of size.

In one experiment whole separated calf thyroid cells were prepared by Dr. Ira Pastan of this Institute using trypsinization. These were labeled *in vitro* by incubation with I^{131} for three hours (31). Following incubation in Krebs-Ringer bicarbonate buffer, the cells were transferred to 0.88 M sucrose solution, homogenized, and the particles surveyed for size by multiple centrifugation as described above. The results show that particulate iodine occurs in three major groups of particles as shown in Figure 7. There is a group of iodinated particles which corresponds exactly with the particles in Fraction I of the tumor tissue. Two other smaller concentrations of iodine containing particles do not correspond exactly with either Fraction II or III of the tumor tissue. In addition to these groups of particles a large amount of particulate iodine precipitated with the lowest force employed.

Hog thyroid glands were obtained at a local abattoir and were sliced thinly by hand. They were incubated for three hours at 37° C with I^{131} in Krebs-Ringer bicarbonate buffer and were then washed (32). The uptake of I^{131} into the slices was 77% of the total I^{131} added to the medium. The slices were then homogenized in sucrose and the whole particulate was isolated and found to contain 6% of the I^{131} taken up by the slices. The particulate was surveyed for the distribution of I^{131}. Good correspondence was found between the groups of iodinated particles in the hog tissue and those in the thyroid tumor. Figure 8 shows the distribution of I^{131} in hog thyroid slices. Half of the I^{131} containing material isolated with

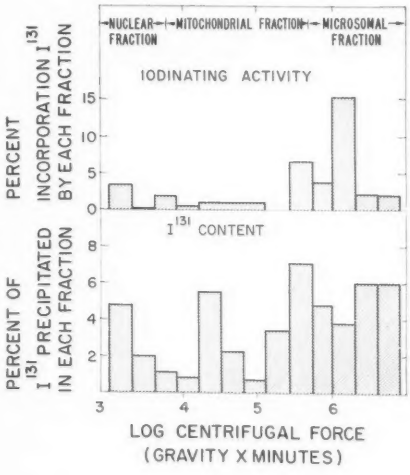

FIG. 8. Iodine content and iodinating activity in hog thyroid particles. The lower part of the figure shows a survey of normal hog thyroid tissue labeled *in vitro* with I^{131}. The correspondence between the iodine containing fractions in this preparation and those in the tumor is striking.

The upper part of the figure shows iodinating activity expressed as percentage of total I^{131} in the iodinating system incorporated into a TCA precipitable form by each fraction. Although the fraction showing the greatest amount of incorporation does not correspond exactly with the major iodine containing fraction, the association of iodine content and iodinating activity in the smaller particles is evident.

the particles could not be precipitated even with the greatest centrifugal force employed. This non-precipitable material was probably soluble iodoprotein which contaminated the pellet. This being the case, only about 3% of the iodine taken up by the hog thyroid slices is actually particulate.

Iodination Experiments Using Cell-Free Particles

Since enzymes responsible for iodination in thyroid homogenates are particulate, it seemed of interest to investigate the fractions of the hog thyroid tissue for iodinating activity. The particulate material was isolated from 2 grams of hog thyroid tissue and fractions were prepared using the survey technique. Suspensions of particles in Krebs-Ringer bicarbonate buffer adjusted to pH 9–10 were used with added flavine adenine dinucleotide (Nutritional Biochemicals), carrier iodide, and I^{131} similar to the system of Taurog and co-workers (5, 15). In most experiments the volume of the system was 2.0 ml. The concentration of FAD was 2×10^{-4}M, and 5 μc of I^{131} and 2 micrograms of potassium iodide were added per 2.0 ml.

The iodinating systems were incubated in 25 ml. Erlenmeyer flasks in a Dubnoff metabolic shaker at 37° for two and a half hours. At the termination of incubation three volumes of 10% trichloroacetic acid were added to each flask and the contents were separated into supernatant and precipitate by centrifugation. The precipitate was washed once with two volumes of 10% TCA, and the supernatants and precipitates were counted to determine the percentage of inorganic I^{131} incorporated into a TCA precipitable form. A complete system as described above was prepared and rapidly heated to 100° C in a boiling water bath to provide a control. All reported results are corrected for the small amount of apparent incorporation of I^{131} observed in the boiled control (1–2% of the total I^{131} present in the system).

The results of this survey for iodinating activity in normal thyroid tissue are shown in Figure 8. Although iodinating activity does not correspond exactly with any single group of iodinated particles in hog thyroid tissue, most of the iodinating activity is precipitated by the same forces that precipitate the two smaller groups of iodine containing particles. There is negligible iodinating activity corresponding to the particles of Fraction I. Taurog *et al.* (5) found that the mitochondrial and microsomal fractions of thyroid tissue were about equally active in the cell-free particulate iodinating system. Our results show that there is activity in both of these fractions although there is more in the microsomes. It is at least possible that the smaller iodine containing particles iodinate themselves.

A similar survey of particulate iodinating activity was attempted using the rat tumor particles, but the fractions collected by the survey technique had no measurable activity. Each tumor, however, consists of several grams of tissue which concentrate iodine to approximately the same extent as a rat thyroid gland. It seems likely that its specific activity in the iodinating system would be much lower than normal thyroid tissue. It is not surprising, therefore, that fractions prepared from a relatively small amount of tissue showed no activity. A much larger amount of particulate from the tumor, which represented one gram of starting tissue, was incubated in 10 ml. of Krebs-Ringer bicarbonate buffer with appropriate amounts of the other components of the iodinating system. A boiled control containing a similar amount of particulate material was prepared, and after incubation for two hours, 5.0% of the I^{131} in the system was incorporated into a TCA precipitable form by the living particles compared with 1.2% incorporated by the boiled control. It appears that the tumor particles are active in the particulate iodinating system; however, this activity is much lower per unit of tissue (or per unit of particulate material) than in normal thyroid tissue.

COMMENTS

The rat thyroid tumor has proved to be a good system for studying the general problem of iodine containing subcellular particles in thyroid tissue.

The large amounts of radioiodine concentrated by the particles and the high radioiodine/nitrogen ratios of the particle fractions containing most of the iodine are evidence against the possibility that the particulate iodine in the tumor is an artefact of subcellular fractionation caused by contamination of the particles by soluble iodoproteins. The general agreement of studies on the distribution of iodine in subcellular particles of the tumor and of thyroid tissue from normal glands is evidence that the observations on thyroid tumor tissue are not intrinsic peculiarities of tumor tissue but represent only an exaggeration of normal conditions.

Taurog *et al.* (5) have shown that cell-free suspensions of subcellular particles oxidize iodine and incorporate it into a particulate iodoprotein. Tong and Chaikoff (16) have shown that the pattern of iodine incorporation into amino acids by particles is similar in intact cells and in cell-free systems. We have shown that some of the particulate fractions containing most of the iodine are the ones which are most active in the cell-free iodinating system. Although one group of iodine containing particles shows no iodinating activity, experiments with the other two groups suggest that the particles are iodinating themselves. This dissimilarity in iodination ability, incidentally, is evidence that Fraction I particles are not simply aggregates of those in Fractions II and III, although it is perhaps possible that such aggregation might affect the iodination reactions. Particles of small size, perhaps comparable to those containing iodine and capable of iodination, have been shown to change in abundance and appearance according to the functional state of the thyroid gland. There is no evidence to date, however, that the iodoprotein of the subcellular particles is at all similar to thyroglobulin or that it bears any precursor-product relationship to it.

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BIOGENESIS OF PROGESTERONE IN OVARIAN TISSUES[1]

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ABSTRACT

Using cholesterol-26-C^{14} and 20α-hydroxycholesterol-22-C^{14} as substrates, the presence of the cholesterol side-chain desmolase in cow ovarian tissues has been demonstrated. The distribution of the desmolase in various ovarian tissues was found to be related to the degree of secretory activity. The conversion of cholesterol-4-C^{14} to progesterone was confirmed in corpus luteum homogenates. The conversion of Δ^5-pregnenolone to progesterone was observed in corpus luteum and, to a lesser extent, in follicular tissue, but not significantly in follicular fluid. Several types of gonadotrohpin failed to increase these reactions *in vitro*.

FOR progesterone production from cholesterol, side-chain-cleaving desmolase activity has been described in ovarian tissue (1), and a 3β-ol-dehydrogenase in the corpus luteum (2). In this paper, the *in vitro* cleavage of cholesterol and of a new intermediate, 20α-hydroxycholesterol (3, 4), and the conversion of pregnenolone to progesterone by ovarian tissue are described.

MATERIALS AND METHODS

Bovine ovaries were collected immediately after slaughter. The corpora lutea were isolated from the other tissues and then homogenized in 0.3 M sucrose solution with a Potter-Elvehjem homogenizer; total ovarian homogenate was obtained with a Waring blendor with the addition of 2.5 parts of 0.3 M sucrose solution. Follicular fluid was collected by syringe at the slaughter house and enough sucrose was added to have the same final concentration as in the total ovarian homogenate. Large follicles were isolated, and after removal of the liquid were homogenized in the sucrose solution. These are designated as follicular tissue homogenates.

The side-chain-cleaving activities in the tissue homogenates were determined by measuring the radioactivity of isocaproic acid obtained as a metabolite of cholesterol-26-C^{14} (5) and 20α-hydroxycholesterol-22-C^{14} (3, 4).

The amount of conversion from Δ^5-pregnenolone to progesterone, or the combined activities of 3β-ol dehydrogenase and isomerase, was determined by measurement of the ultraviolet absorption at 240 mμ due to the progesterone produced. The progesterone was isolated by a column chromatographic separation with silica gel, and then identified by paper chromatography. Correction factors were calculated for the chromatographic ex-

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tracts from the optical densities at 220 mμ, 240 mμ, and 260 mμ, and the progesterone content determined by reference to a standard progesterone curve.

EXPERIMENTAL RESULTS

Cholesterol as Substrate

Cleavage of cholesterol side-chain into isocaproic acid

Five ml. of corpus luteum homogenate, 2.5 ml. of 0.3 M Tris buffer (pH 8.1), 0.002 M DPN, 0.002 M ATP, and 0.02 M $MgCl_2$ were added to flasks containing cholesterol-26-C^{14} (310,355 cpm per flask) and incubated aerobically for 3 hr at 38° C in a Dubnoff shaker. The radioactivity of the isocaproic acid fractions isolated (after the method of Shimizu *et al.* (3)) from the mixture was expressed as counts per minute (cpm) corrected for background, using the Packard Tri-Carb Liquid Scintillation Spectrometer (Table 1). The clear increase in side-chain cleavage indicated is not significantly affected by the addition of chorionic gonadotrophin.

TABLE 1. CLEAVAGE OF THE CHOLESTEROL SIDE CHAIN IN CORPUS LUTEUM HOMOGENATES

Treatment	Radioactivity of isocaproic acid (cpm)		
	Exp. 1	Exp. 2	Exp. 3
Before incubation	292	714	120
Incubated without gonadotrophin	1464	2966	3157
Incubated with gonadotrophin*	1458	2572	2481

* Human chorionic gonadotrophin (A.P.L.) 2,000 I.U. per flask.

Conversion of cholesterol-4-C^{14} to progesterone

Ten μc of cholesterol-4-C^{14} was incubated with cow corpus luteum homogenate in a 20 ml. volume under the same conditions as in the previous experiment. Incubation was stopped after 3 hr. by acidification with HCl. Before the incubation, 28 mg. of non-radioactive progesterone was added as a carrier for isolation. The homogenate was extracted with 100 ml. isopropyl acetate three times, and then once with 325 ml. of acetone. The pooled extracts were dried, taken up in ethanol, and treated with digitonin. They were kept overnight in a refrigerator. The ethanol-washed digitonide precipitate was separated from the supernatant. After evaporating the supernatant to dryness, the residue (about 3% of the pooled extract radioactivity) was extracted with ether. The ether soluble fraction was evaporated to dryness and the residue was transferred to a silica gel column and chromatographed with mixtures of ethyl acetate in benzene. An aliquot of ethyl acetate:benzene, 1:9, eluate was chromatographed on paper with the cyclohexane-formamide system. The spot which corresponded to authentic progesterone run as a standard reference on a parallel limb, was eluted with methylene chloride. This eluate was rechromatographed in the same system. The radioactive peak found by scanning the chromatogram

TABLE 2. THE RADIOACTIVITY OF THE PROGESTERONE OBTAINED FROM CHOLESTEROL-4-C^{14} AFTER RECRYSTALLIZATION

No. of recrystallization	Radioactivity (cpm)	Progesterone (mg.)	Specific activity (cpm/mg.)
1st	1341	1.650	813
2nd	892	1.075	829
3rd	689	0.845	815

paper corresponded exactly with the location of progesterone as determined by reference to a progesterone standard which was simultaneously chromatographed on a parallel limb. The radioactive spot thus located was eluted and rechromatographed on silica gel. The radioactive eluate from this column chromatogram was diluted with authentic progesterone (22 mg.) as carrier, and was recrystallized three times from petroleum ether:acetone mixtures. The specific activities for each crystallization: 813, 829, and 815 cpm/mg., suggest the radioactive purity of the progesterone produced (see Table 2).

20α-Hydroxycholesterol-22-C^{14} as substrate

Cleavage of the side chain as measured by the radioactivity of isocaproic acid in the corpus luteum homogenates

20α-Hydroxycholesterol (81, 880 cpm per flask) was incubated with the homogenate under the conditions previously described. In Table 3 the cleavage is expressed in counts per minute of the radioactive isocaproic acid produced from the substrate. Desmolase activity in the incubates is clearly indicated by the data of Table 3. While the two flasks containing HCG showed small increases in isocaproic acid over the controls, the number of experiments is too small to determine its significance. Certainly in the case of FSH and PMS, there was no evidence of any effect of these gonadotrophins on side chain cleavage.

Desmolase activity in ovarian tissues

Whole ovaries were homogenized in the Waring blendor with addition of 2.5 parts of 0.3 M sucrose solution. The homogenate (5 ml.) was incu-

TABLE 3. THE CLEAVAGE OF THE SIDE CHAIN OF 20α-HYDROXYCHOLESTEROL-22-C^{14} IN THE CORPUS LUTEUM HOMOGENATE

Treatment	Isocaproic acid radioactivity (cpm)				
	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5
Before incubation	240	301	123	126	62
Incubation without gonadotrophin	8,149	4,977	4,271	6,518	6,532
Incubation with gonadotrophin	11,072*	6,358*	4,292*	5,208†	5,743‡

* Human chorionic gonadotrophin (A.P.L.) 2,000 I.U. per flask.
† Purified FSH 1 mg. per flask, supplied by National Institutes of Health.
‡ Pregnant mare serum gonadotrophin 250 I.U. per flask.

TABLE 4. DESMOLASE ACTIVITY IN OVARIAN TISSUE COMPONENTS

Side chain cleavage of 20α-hydroxycholesterol-22-C^{14} (81,800 cpm) by homogenates of whole ovaries with and without corpora lutea, in 0.3 M sucrose.

Exp. No.	Description of ovary			Radioactivity of isocaproic acid (cpm)	Remarks	
	Weight (gm.)	Weight of corpus luteum (gm.)	Follicle		pH of medium	Without incubation* (cpm)
1	12.9	5.0	Large one, 0.8 cm. in diameter	4,996	8.1	284
	6.6	none	Scattered small follicles	2,051	8.1	308
2	14.6	7.2	Large one, 1.6 cm. in diameter	7,044	8.1	168
	11.9	none	Large follicle, 1.8 cm. in diameter	6,683	8.1	153
3	15.0	6.6	Only small and scattered follicles	30,166	8.3	
	6.3	none	Only small and scattered follicles	3,320	8.3	
4	15.1	7.5	Only small and scattered follicles	35,185	8.3	
	9.8	none	Large follicle, 2.0 cm. in diameter	6,616	8.3	
5			Follicle fluid	738 1,021	8.3 8.3	
			Follicular tissue homogenate	14,170 12,876	8.3 8.3	

* Counts of isocaproic acid obtained immediately after addition of substrate to a tissue aliquot which was then processed like the incubates.

bated with the 20α-hydroxycholesterol as in the previous experiments. Table 4 describes the data obtained with corpus-luteum-containing and corpus-luteum-free tissue (Experiments 1, 2, 3, 4) and with the fluid of follicles the tissues of which were simultaneously homogenized (Experiment 5). These data demonstrate the greater activity in corpus-luteum-containing preparations. They indicate also minimal or negligible desmolase activity in follicular fluid, equivalent to 5% to 8% of the tissue of the follicle.

Conversion of Pregnenolone to Progesterone in Ovarian Tissue

Pregnenolone (Δ^5-pregnene-3β-ol-20-one), 10 mg. per flask, incubated with 5 ml. of corpus luteum homogenate as in previous experiments

Table 5 presents the yields of progesterone from homogenate samples taken before incubation and following incubation with and without added Δ^5-pregnenolone. The data demonstrate: (1) undetectable amounts of pro-

TABLE 5. CONVERSION OF Δ^5-PREGNENOLONE TO PROGESTERONE IN CORPUS LUTEUM HOMOGENATE

Treatment	μg of progesterone produced	
	Experiment 1	Experiment 2
Before incubation	0	0
Incubated without pregnenolone	93.4*	70.8*
Incubated with pregnenolone	960. *	802. *

* Identical with progesterone by paper chromatography.

gesterone in the preincubation samples, (2) a small but definite yield when pregnenolone is not added, and (3) an increased yield in the samples to which Δ^5-pregnenolone was added.

Isolation of progesterone after incubation of Δ^5-pregnenolone with corpus luteum homogenates

50.0 Mg. of Δ^5-pregnenolone were incubated with corpus luteum homogenates (10 g in 25 cc.) in Krebs-Ringer bicarbonate buffer (pH 7.4) plus glucose (100 mg.%) with added ATP and DPN (final concentration 0.002 M respectively) for 3 hours in a Dubnoff shaking incubator. HCl was then added to stop the incubation, and the acidified mixture was extracted with isopropyl acetate three times, 200 ml., 200 ml., and 100 ml. respectively. After the solvent had been removed under reduced pressure, the isopropyl acetate extract (291.19 mg.) was chromatographed on a silica gel column using benzene and benzene:ethyl acetate mixtures as eluants. After ultraviolet absorption analysis, it was found that compounds absorbing at around 240 mμ are mainly in the eluate benzene:ethyl acetate 9:1, and a little in the eluate benzene:ethyl acetate 13:7. The analysis showed that the former fraction contained α, β unsaturated ketone equivalent to 3.1 mg. of progesterone (6.2% in yield). An aliquot of the 9:1 benzene:ethyl acetate eluate when chromatographed on paper resulted in a spot which ran as progesterone, absorbed ultraviolet light, and was positive to the isonicotinic hydrazide reagent. The spot was eluted and an aliquot was mixed with progesterone-C^{14} (ca. 8,000 cpm) and rechromatographed first in the cyclohexane-formamide system and then in the propylene glycol-toluene system. The radioactive peak was found to coincide with the spot found by ultraviolet light absorption and isonicotinic acid hydrazide reagent and also with the spot found with authentic progesterone-C^{14} run simultaneously on separate paper strips.

Conversion of Δ^5-pregnenolone by other ovarian tissues

Follicle fluid and follicle tissue homogenates were separately incubated with pregnenolone. With follicle fluid, no conversion could be detected. However, the follicle tissue homogenate showed a very small production

of an α,β-unsaturated ketone (28μg) which was identified by paper chromatography as progesterone.

Influence of gonadotrophin upon the conversion

Gonadotrophic preparations such as human chorionic gonadotrophin, pregnant mare's serum gonadotrophin, purified sheep FSH and LH alone or combined, and purified prolactin did not yield any significant increase in progesterone production from Δ^5-pregnenolone by ovarian tissue homogenates.

DISCUSSION

The conversion steps from cholesterol to pregnenolone and progesterone by the adrenal subcellular fraction have been discussed by Shimizu *et al.* (3, 4). In this paper we have demonstrated corpus luteum and follicular tissue homogenates to be steroidogenic by their capacity to cleave the side chain of cholesterol or of 20α-hydroxycholesterol as in the case of adrenal preparations. Also, it was found that cholesterol could be converted to progesterone by corpus luteum homogenate. Using the 20α-hydroxycholesterol as substrate, desmolase activities were found to be concentrated in corpus-luteum-carrying ovaries, and present only to a very limited extent in corpus-luteum-free ovaries. Pregnenolone, the most probable metabolite of cholesterol cleavage, was also found to be converted to progesterone by corpus luteum homogenates. However, follicular tissue homogenates showed very little such enzymic activity, and in follicular fluid none was detectable (2), although progesterone has been measured in follicular fluid (7). Because corpus-luteum-free ovaries would have a much higher component of connective tissue than would ovaries carrying corpora lutea, the limited enzymatic activity shown by follicle tissue homogenates may have been due to the smaller amount of secretory material represented.

As for gonadotrophin activity upon steroidogenesis, there have been reported: (a) the accumulation of Δ^4-androstenedione-3, 17 in rat diestrus ovaries following administration of pregnant mare serum gonadotrophin (8), (b) the stimulation of production of radioactive testosterone from acetate-C^{14} under the influence of human chorionic gonadotrophin (9), and (c) the acceleration of estrogen formation from testosterone by follicle stimulating hormone (10). However, gonadotrophin and other pituitary hormones have been reported as incapable of increasing *in vitro* progesterone production in corpus luteum slice incubates without precursor (11), and the *in vitro* administration of chorionic gonadotrophin to testes did not change the 3β-ol dehydrogenase activity (12). Experiments on the perfusion of C^{14}-acetate through corpus-luteum-carrying ovaries have suggested an increase of progesterone synthesis when crude sheep gonadotrophin is administered (13). Intact tissue may, therefore, be necessary

for the gonadotrophin effect or certain specific conditions (not met in the foregoing experiments) of gonadotrophin combination and addition to incubate may be requisite.

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INHIBITORY EFFECT OF VARIOUS STEROIDS ON GONADOTROPHIN HYPERSECRETION IN PARABIOTIC MICE[1]

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ABSTRACT

A variety of steroids have been administered subcutaneously or orally into the castrated male partner of parabiotic mice to investigate their anti-gonadotrophic activity based on the inhibition of ovarian growth in the intact partner united with the castrated male.

The data demonstrate that estrogenicity is most likely responsible for anti-gonadotrophic activity of the compound, but either androgenic or progestational potency of the steroid is not always proportional to its antipituitary action. According to the compound, especially in 19-norsteroids, the oral activity on the gonadotrophin inhibition is not always parallel with the subcutaneous potency. Norethynodrel and norethisterone, both of which are known as orally active anti-ovulatory agents, have roughly the same order of anti-gonadotrophic activity as that of estrone in the oral test. However, 6-methyl-17-acetoxyprogesterone, 17-1.methallyl- or 17-2.methallyl-19-nortestosterone, all of which are orally potent progestational substances, like progesterone itself do not show any significant inhibition of pituitary gonadotrophin when administered orally.

Further, as a possible mechanism for the manifestation of anti-gonadotrophic action of various steroids, it is discussed that a part of the compound may convert into some estrogen-like substance in the body, the amount of which is able to inhibit gonadotrophin secretion but not enough to affect peripheral targets, although a major part of the steroid administered is responsible for the hormonal activities the compound may have.

BESIDES the naturally occurring sexagens, a number of synthetic steroids have recently been demonstrated as anti-gonadotrophic substances which inhibit the secretion of pituitary gonadotrophin (1, 2, 3, 4, 5). Most of these experiments have been carried out using parabiotic rats.

In our previous paper (6), it was demonstrated that parabiotic mice are useful for the estimation of gonadotrophin hypersecretion and its inhibition by certain steroids, and that a low mortality of parabionts was observed even in the use of non-littermate mice. Subsequently, in our laboratory, the

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anti-gonadotrophic activity of various compounds has been tested in parabiotic mice to investigate whether the pituitary gonadotrophin inhibiting activity of the synthetic compounds is proportional to peripheral effects such as estrogenic, progestational, androgenic or anabolic activity.

The steroids used in this experiment are as follows: estrone, estriol, ethynyl-estradiol, ethynyl-estradiol-3-methyl-ether, diethylstilbestrol, methyltestosterone, methylandrostenediol, ethynyltestosterone, 2α-methyl-dihydrotestosterone, 2α,17α-dimethyl-dihydrotestosterone, 2-hydroxymethylene-17α-methyldihydrotestosterone, Δ^4-pregnenolone, progesterone, 17α-acetoxyprogesterone, 6α-methyl-17α-acetoxyprogesterone, 17α-ethyl-19-nortestosterone (norethandrolone), 17α-ethynyl-19-nortestosterone (norethisterone), 17α-ethynyl-5(10)-estraenolone (norethynodrel), 17α-1. methallyl-19-nortestosterone, 17α-2.methallyl-19-nortestosterone, cortisone acetate, hydrocortisone acetate and desoxycorticosterone acetate.

METHODS

1. *Anti-gonadotrophic activity*

Non-littermate albino mice, weighing 15 to 17 gm. of our inbred colony (ddS-strain) were used. The castrated male and intact female were put into parabiosis by a modification of the Bunster and Meyer (7) method under methylhexabarbital sodium anesthesia as previously described (6).

Steroids, as fine crystal suspensions, were injected subcutaneously or force fed by gavage into the castrated partner once daily for 10 days starting on the day of parabiotic union. The suspending vehicle consists of an aqueous solution of sodium chloride (0.9%), polysorbate 80 (0.4%), carboxymethyl-cellulose (0.5%) and benzyl alcohol (0.9%). The volume injected or fed was 0.1 ml. per day.

Two control groups of parabionts, castrated male-intact female and non-castrated male-intact female, the male partner of which received suspending vehicle only, were set up for estimating the inhibition of gonadotrophic secretion by the steroidal compounds. All pairs were sacrificed on the day following the last treatment, and gonadal organs were weighed. The percentage inhibition of ovarian growth calculated according to the following formula served as an indicator of gonadotrophin inhibition; $100\times$ (V-C)/(V-Vi), where V, C and Vi indicate, respectively, the average ovarian weights of the intact female united with the vehicle-injected castrated male, the compound-injected castrated male and the vehicle-injected non-castrated male.

2. *Uterotrophic activity*

Uterotrophic activity was determined in 8 to 10 gm. immature female mice (ddS-strain). The steroids dissolved in sesame oil were administered subcutaneously once daily for 3 consecutive days to dose groups of 10 or 20 animals. The animals were sacrified on the day following the last injection. The uteri were weighed after the fluid was expressed.

3. *Androgenic and myotrophic activity.*

A modification of the Eisenberg and Gordon method (8) was used. Wistar male rats weighing between 40 and 50 gm. were castrated and divided into dosage groups of 10 or more animals each. Starting 48 hours after castration, the compounds were adminstered subcutaneously as fine crystal suspensions once a day for 7 consecutive days. The

composition of suspending vehicle was the same as that used in the anti-gonadotrophic activity test. The animals were sacrified on the following day and seminal vesicles, ventral prostate and levator ani muscles removed and weighed. Fluid was expressed from the seminal vesicles prior to weighing.

RESULTS

1. *Anti-gonadotrophic activity*

a) Reproducibility of the castration hypersecretion.

Before estimating the steroid inhibition of gonadotrophin secretion, the reproducibility of castration-hypersecretion of gonadotrophin in the parabiotic mice was confirmed. Each of the six technical assistants made 3 or 4 pairs of intact male-female and the same pairs of castrated male-female on the same day. Only the suspending vehicle 0.1 ml. per day was injected subcutaneously once daily for 10 days into the male partner.

The data on 7 sets of experiments were analyzed, as presented in Table 1,

TABLE 1. REPRODUCIBILITY OF CASTRATION-HYPERSECRETION OF GONADOTROPHIN

a. Intact male-female parabionts

Date	Mortality	No. of pairs	Ovarian weight*	
			Mean	±S.E.
Jan. 27	0/15	15	51.3	±2.8
Feb. 3	4/18	14	44.1	±3.1
Feb. 10	6/18	12	39.4	±2.5
Feb. 16	3/19	16	31.8	±1.3
Mar. 2	3/18	15	38.3	±1.9
Mar. 9	1/19	18	34.6	±1.9
Mar. 16	0/18	18	35.9	±2.2
Operator				
A	4/21	17	33.0	±1.9
B	2/22	20	39.2	±1.9
C	2/21	19	39.9	±2.6
D	3/22	19	41.5	±2.2
E	1/18	17	36.4	±2.0
F	5/21	16	43.8	±3.7

b. Castrated male-female parabionts

Date	Mortality	Failure	No. of pairs	Ovarian weight*	
				Mean	±S.E.
Jan. 27	1/15	0/14	14	113.4	± 6.3
Feb. 3	2/18	1/16	15	108.7	± 7.1
Feb. 10	3/18	2/15	13	105.0	± 6.7
Feb. 16	1/19	6/18	12	85.5	± 5.5
Mar. 2	3/19	5/16	11	108.0	± 6.8
Mar. 9	1/19	5/18	13	99.9	±11.9
Mar. 16	0/19	2/19	17	129.7	±11.7
Operator					
A	2/21	6/19	13	101.5	± 8.1
B	2/23	2/21	19	114.0	± 7.3
C	1/21	2/20	18	94.5	± 3.6
D	2/22	2/20	18	116.7	±11.4
E	1/18	4/17	13	112.5	± 7.2
F	3/22	5/19	14	110.8	±10.8

* mg. per 100 gm. body weight.
A, B, C, D, E, and F: person engaged in the operation.
Failure: the data (ovarian weight) below 60 mg. per 100 gm., indicating the failure of gonadotrophin-hypersecretion.

for the mortality of parabionts, failure of gonadal hypertrophy (number of pairs failing to show any gonadal stimulation in the intact partner) and mean ovarian weight with standard error, according to the date of operation and the individual operators. The data indicate fair reproducibility of gonadotrophin hypersecretion and relative homogeneity of parabiosis technic of the six operators.

b) Subcutaneous assay.

The data on the groups treated by estrogens, androgens, gestagens and corticoids are presented in Tables 2, 3, 4 and 5, respectively.

TABLE 2. ANTI-GONADOTROPHIC ACTIVITY OF ESTROGENS (SUBCUTANEOUS ASSAY)

Compound	Total dose (mg.)	No. of pairs	Castrated		Intact		% inhibition of ovarian growth
			Seminal vesicles	Ventral prostate	Uterus	Ovaries	
Estrone	10^{-4}	4	92±18.3*	10±1.0*	91± 8.4*	35± 3.3*	108
	10^{-5}	4	56± 9.4	9±1.2	121± 28.7	42± 4.4	99
	10^{-6}	8	80±15.7	11±0.9	474± 49.9	101±10.2	15
	10^{-7}	5	85±12.7	11±1.3	571± 43.7	109± 6.6	4
Estriol	10^{-2}	5	77±10.0	9±1.8	200± 67.6	46± 5.9	93
	10^{-3}	4	76±11.1	12±1.6	559±140.5	112± 8.6	0
	10^{-4}	5	69±10.0	12±2.7	403± 96.3	122±17.3	−14
Ethynylestradiol	10^{-3}	5	72± 8.5	10±2.1	125± 9.3	44± 3.6	96
	10^{-4}	6	61± 2.1	10±0.8	128± 23.1	47± 7.0	92
	10^{-5}	6	85±25.6	14±1.6	445± 91.0	100±26.8	17
	10^{-6}	5	82±12.7	12±1.0	665± 63.5	106± 9.0	8
17α-Ethynylestradiol-3-methyl-ether	10^{-3}	5	79± 4.6	11±1.0	159± 36.7	43± 5.1	97
	10^{-4}	4	68± 9.1	11±0.4	250±113.3	39± 5.8	103
	10^{-5}	5	94±12.2	9±1.2	541± 84.6	116±19.7	−6
	10^{-6}	5	69± 2.9	14±1.3	567±104.7	115±16.2	−4
Diethylstilbestrol	10^{-3}	4	84± 8.1	10±1.0	98± 7.6	47± 7.0	92
	10^{-4}	5	72±12.0	9±1.3	78± 9.2	46±10.2	93
	10^{-5}	5	85±11.9	8±1.2	367±139.2	100±18.7	17
	10^{-6}	5	69± 9.6	8±1.2	448±149.4	107±10.1	7
	10^{-7}	4	58±10.7	14±1.7	625± 86.8	121±10.3	−13
Control (castrated male-female)		18	82± 7.3	9±0.9	590± 43.8	122± 4.4	
(intact male-female)		9	165±30.7	23±4.5	115± 6.1	41± 3.6	

* Mean ± S.E. (mg. per 100 gm. body weight).

All estrogens (estrone, estriol, ethynyl-estradiol and its 3-methylether and stilbestrol) injected into the castrated male inhibit the gonadal hypertrophy of intact female partner. Estrone is the most active and estriol the weakest in this group. The other three estrogens (stilbestrol, ethynyl-estradiol and its 3-methylether) are intermediate and their potencies are almost identical.

Six androgens (methyltestosterone, methylandrostenediol, ethynyltestosterone, 2α-methyl-, 2α,17α-dimethyl- and 2-hydroxymethylene-17α-methyl-dihydrotestosterone) are all active in this test, but they are less potent than most of the estrogens. Methyltestosterone, methylandrostenediol and ethynyltestosterone induce almost the same order of gonadotrophin inhibition as that of estriol without any exertion of androgenic effect on the castrated male partner. The three dihydrotestosterones are less active than the former three androgens, and their anti-gonadotrophic effects are accompanied with marked androgenic action on the castrated male partner, as indicated in Table 3.

TABLE 3. ANTI-GONADOTROPHIC ACTIVITY OF ANDROGENS (SUBCUTANEOUS ASSAY)

Compound	Total dose (mg.)	No. of pairs	Castrated		Intact		% inhibition of ovarian growth
			Seminal vesicles	Ventral prostate	Uterus	Ovaries	
Methyltestosterone	10^{-1}	6	62±19.3*	10±0.7*	153± 30.1*	42± 3.4*	99
	10^{-2}	5	79±15.9	12±1.6	246± 89.4	52±11.8	85
	10^{-3}	4	63± 7.9	8±2.5	592± 78.5	112±15.5	0
Methylandrostenediol	10^{-1}	5	86± 9.9	13±1.7	325± 83.8	56± 8.4	79
	10^{-2}	5	67±13.3	7±1.4	202± 92.1	51± 7.3	86
	10^{-3}	5	75± 7.2	12±0.6	546± 72.0	117±15.7	−7
Ethynyltestosterone	10^{-1}	4	74±13.3	14±0.9	257± 61.9	57± 8.3	77
	10^{-2}	6	84±17.9	13±1.1	347±104.9	59±10.9	75
	10^{-3}	4	89±17.0	10±2.9	545± 32.6	102±16.8	14
2α-Methyldihydrotestosterone	10	5	397±38.5	43±1.9	89± 8.5	33± 3.3	111
	1	6	239±19.2	18±0.9	90± 5.1	31± 1.0	114
	10^{-1}	6	100±12.5	13±2.6	549± 52.6	117±10.9	−7
2α,17α-Dimethyldihydrotestosterone	1	6	327±32.2	30±5.0	88± 12.0	36± 4.2	107
	10^{-1}	9	83± 5.1	9±1.1	546± 45.9	102± 6.6	14
	10^{-2}	5	62± 6.5	13±1.0	667± 28.2	124±10.3	−17
2-Hydroxymethylene-17α-methyl-dihydrotestosterone	1	6	266±15.0	21±4.0	89± 10.9	41± 2.9	100
	10^{-1}	6	90± 2.5	14±6.0	544± 88.7	108±13.4	6
	10^{-2}	4	96± 9.3	11±1.3	520± 32.1	110±16.3	3
Control (castrated male-female)		18	82± 7.3	9±0.9	590± 43.8	112± 4.4	
(intact male-female)		9	165±30.7	23±4.5	115± 6.1	41± 3.6	

* Mean ± S.E. (mg. per 100 gm. body weight).

TABLE 4. ANTI-GONADOTROPHIC ACTIVITY OF GESTAGENS (SUBCUTANEOUS ASSAY)

Compound	Total dose (mg.)	No. of pairs	Castrated		Intact		% inhibition of ovarian growth
			Seminal vesicles	Ventral prostate	Uterus	Ovaries	
Δ^5-Pregnenolone	10	6	118±16.3*	20± 1.3*	396±147.4*	89±15.9*	32
	1	7	123±12.2	10± 0.7	594± 63.6	119±10.5	−10
	10^{-1}	5	76± 7.3	14± 1.3	583± 84.2	134±16.9	−31
Progesterone	10	6	104±21.6	14± 2.2	99± 15.6	33± 3.9	111
	1	6	75± 9.5	10± 1.3	365± 99.4	86±16.3	37
	10^{-1}	4	47± 3.8	5± 1.2	589± 68.8	139±10.0	−38
	10^{-2}	5	77± 8.2	9± 0.9	579±112.1	125±16.4	−18
17α-Acetoxyprogesterone	1	4	118±21.9	17± 2.7	94± 14.8	38±12.4	104
	10^{-1}	4	66± 3.8	7± 0.9	574± 46.1	104±11.9	11
6α-Methyl-17α-acetoxyprogesterone	10^{-2}	5	87±12.7	11± 1.5	308± 44.9	50± 9.5	87
	10^{-3}	5	112±19.8	10± 0.8	201± 72.2	47±10.0	92
	10^{-4}	4	116±14.7	13± 1.5	236± 51.4	68± 9.4	62
	10^{-5}	4	57± 5.2	6± 0.8	562± 18.9	115± 9.5	−4
17α-Ethyl-19-nortestosterone	1	4	161±21.9	12± 0.5	122± 5.5	35± 2.0	108
	10^{-1}	6	76± 5.1	9± 1.3	205± 70.6	44± 5.2	96
	10^{-2}	5	110±13.7	12± 2.1	494± 69.4	89± 9.4	32
	10^{-3}	5	81± 7.9	18± 2.2	477± 75.8	113±12.4	−1
17α-Ethynyl-19-nortestosterone	10	4	237±38.9	27± 3.8	414± 46.1	56± 2.3	79
	1	5	235±12.5	24± 2.5	303± 36.4	51± 4.1	86
	10^{-1}	6	85± 4.5	13± 1.5	111± 5.3	57± 3.0	77
	10^{-2}	6	78±11.7	11± 1.1	397± 96.4	90±10.5	31
	10^{-3}	6	70±13.1	10± 1.3	456± 5.7	114± 3.8	−3
17α-Ethynyl-5(10)-estraenolone	10^{-1}	5	95±12.8	15± 1.3	196± 55.0	52± 8.0	85
	10^{-5}	5	98±15.3	10± 1.7	362±107.2	78±22.6	48
	10^{-6}	6	94±11.6	8± 0.9	323± 53.5	84±10.4	39
	10^{-7}	5	118±13.5	12±10.8	567± 74.6	120±11.4	−11
17α-1.Methyallyl-19-nortestosterone	1	8	101±10.2	10± 0.9	201± 39.2	47± 4.0	92
	10^{-1}	5	64± 6.2	9± 1.2	553± 19.0	96±11.6	23
17α-2.Methallyl-19-nortestosterone	1	4	92± 6.3	13± 1.3	295± 63.8	62±10.1	70
	10^{-1}	6	77± 8.8	11± 1.0	585± 62.5	106±12.7	8
Control (castrated male-female)		18	82± 7.3	9± 0.9	590± 43.8	112± 4.4	
(intact male-female)		9	165±30.7	23± 4.5	115± 6.1	41± 3.6	

* Mean ± S.E. (mg. per 100 gm. body weight).

The data on progestational steroids (Table 4), except for the 19-norsteroids, suggest that the anti-gonadotrophic activity of the compound is roughly related to its progestational activity as estimated in the Clauberg rabbit (9). The most potent progestin in this group, 6-methyl-17-acetoxyprogesterone, shows marked inhibition of ovarian growth, the grade of which is between estrone and estriol, without significant androgenic reaction in the castrated male. Among the 19-norsteroids, however, the order of anti-gonadotrophin activity is not always parallel with that of progestational potency as indicated in Figure 4. 17-2.Methallyl-19-nortestosterone, an extremely potent progestational substance in the Clauberg assay (10), does not show any superiority in the anti-gonadotrophin test. On contrast, a weak progestin, norethynodrel, shows marked gonadotrophin suppres-

TABLE 5. ANTI-GONADOTROPHIC ACTIVITY OF CORTICOIDS (SUBCUTANEOUS ASSAY)

Compound	Total dose (mg.)	No. of pairs	Castrated		Intact		% inhibition of ovarian growth
			Seminal vesicles	Ventral prostate	Uterus	Ovaries	
Cortisone acetate	1	5	70±11.5*	16±4.0*	648± 88.4*	120±15.8*	−11
	10^{-1}	6	57± 8.4	12±1.5	634± 61.7	132± 6.5	−28
Hydrocortisone acetate	1	5	75± 7.0	15±1.5	502±126.4	118±21.6	− 8
	10^{-1}	4	75± 9.8	9±0.2	556±160.5	125±30.5	−18
Desoxycorticosterone acetate	10	6	78±11.1	9±0.9	299± 68.1	85±15.4	38
	1	4	55± 8.8	6±0.5	453± 63.5	100± 9.5	17
	10^{-1}	5	66± 7.8	8±1.1	429±150.4	96±18.9	23
Control (castrated male-female)		18	82± 7.3	9±0.9	590± 43.8	112± 4.4	
(intact male-female)		9	165±30.7	23±4.5	115± 6.1	41± 3.6	

* Mean ± S.E. (mg. per 100 gm. body weight).

sion, the activity of which is equivalent to that obtained by ethynylestradiol. However, small amounts of the aromatic steroids, mainly ethynyl-estradiol-3-methylether, contaminating this compound might account for a part of this activity. A large dose of norethandrolone and norethisterone produces androgenic effects along with the anti-gonadotrophic effect, but the thresholds of their effective doses for gonadotrophin inhibition are lower than that for male accessory organ stimulation.

As indicated in Table 5, desoxycorticosterone acetate has a slight gonadotrophin inhibiting activity, but neither cortisone acetate nor hydrocortisone acetate inhibits pituitary gonadotrophin hypersecretion in parabiotic mice in a total dose up to 10 mg. per pair.

c) Oral assay.

The results obtained by oral administration of the steroids are presented in Tables 6 and 7. Compared with the subcutaneous test, all steroids showed a reduced activity.

By oral admlnistration, estrone markedly reduces its anti-gonadotrophic activity, whereas ethynyl-estradiol-3-methylether does not. The oral activities of androgens are approximately 1/10 to 1/100 of their subcutaneous activities. Almost complete inhibition of gonadotrophin hypersecretion

TABLE 6. ANTIGONADOTROPHIC ACTIVITY OF ESTROGENS AND ANDROGENS (ORAL ASSAY)

Compound	Total dose (mg.)	No. of pairs	Castrated		Intact		% inhibition of ovarian growth
			Seminal vesicles	Ventral prostate	Uterus	Ovaries	
Estrone	10^{-1}	7	63± 4.1*	7±0.2*	228± 41.7*	54± 6.0*	71
	10^{-2}	7	49± 6.0	7±0.9	530± 79.9	85±10.0	19
	10^{-3}	13	43± 3.8	8±0.8	571± 50.4	105±10.2	−15
17α-Ethynylestradiol-3-methyl-ether	10^{-2}	6	43± 2.6	7±0.6	103± 4.9	37± 2.7	100
	10^{-3}	6	31± 4.5	5±0.6	127± 30.0	29± 4.0	114
	10^{-4}	6	35± 3.3	6±1.1	377±100.7	66±12.5	51
Testosterone propionate	10	6	131±13.7	26±3.2	139± 35.1	44±13.0	88
	1	6	39± 4.2	12±0.6	441±122.1	95±13.9	2
Methyltestosterone	10	7	115±10.6	21±1.5	245± 77.0	49± 5.8	80
	1	14	53± 4.1	14±0.9	309± 56.6	63± 6.9	56
	10^{-1}	6	38± 6.9	11±0.4	372±107.8	79±17.8	29
	10^{-2}	6	38± 4.2	10±1.0	365±101.5	76±20.0	34
Ethynyltestosterone	10	7	40± 8.7	10±1.2	306± 78.1	66± 8.5	51
	1	6	27± 4.8	7±1.2	386± 74.9	79±14.2	29
2-Hydroxymethylene-17α-methyl-dihydrotestosterone	10	5	102±10.5	22±1.6	132± 22.3	42± 8.1	92
	1	5	48± 4.2	13±1.4	284±113.0	58±16.0	64
Control (castrated male-female)		59	33± 1.5	7±0.3	432± 26.7	96± 5.2	
Control (intact male-female)		38	52± 4.7	14±0.8	111± 13.5	37± 1.6	

* Mean ± S.E. (mg. per 100 gm. body weight).

produced by these androgens is always accompanied with the stimulation of male accessory organs.

Progesterone, 6-methyl-17-acetoxyprogesterone, and 17-1.methallyl or 17-2.methallyl-19-nortestosterone produce no significant inhibition of

TABLE 7. ANTI-GONADOTROPHIC ACTIVITY OF GESTAGENS (ORAL ASSAY)

Compound	Total dose (mg.)	No. of pairs	Castrated		Intact		% inhibition of ovarian growth
			Seminal vesicles	Ventral prostate	Uterus	Ovaries	
Progesterone	10	7	25± 1.2*	5±0.4*	442± 76.6*	85±14.9*	19
	1	6	23± 1.4	6±1.2	407±120.2	80±18.6	27
	10^{-1}	7	26± 1.9	7±0.7	380± 93.7	84±12.8	20
6α-Methyl-17α-acetoxy-progesterone	10	5	31± 5.2	9±1.6	439±147.5	90±18.5	10
	1	7	30± 2.5	8±0.7	444± 65.6	99±11.9	−5
	10^{-1}	13	45± 6.0	8±0.7	344± 53.4	79±10.1	29
	10^{-2}	5	53± 9.7	10±1.0	420±133.3	79±14.5	29
	10^{-3}	6	52± 7.0	7±0.8	401±107.8	80±12.2	27
17α-Ethyl-19-nortesto-sterone	10	7	72±11.3	11±1.5	103± 13.3	38± 3.2	98
	1	6	50± 6.9	9±1.0	89± 6.1	37± 3.3	100
	10^{-1}	7	46± 4.4	8±0.7	149± 28.4	70±15.4	44
17α-Ethynyl-19-nortesto-sterone	10	6	56± 7.6	8±1.2	172± 12.4	38± 3.0	98
	1	7	34± 3.1	8±0.9	143± 29.1	37± 4.7	100
	10^{-1}	13	31± 2.9	7±0.5	190± 36.8	56± 7.8	68
	10^{-2}	6	33± 5.0	9±0.5	211± 71.9	61±13.2	59
	10^{-3}	6	30± 0.9	7±0.8	312±100.2	73±15.5	39
17α-Ethynyl-5(10)-estra-enolone	10	5	47± 2.6	11±1.5	482± 59.7	37± 3.7	100
	1	6	42± 3.8	9±0.4	274± 45.9	39± 3.9	97
	10^{-1}	6	42± 3.6	8±0.9	108± 21.7	41± 4.3	93
	10^{-2}	11	35± 6.0	7±0.5	333± 63.0	69±13.4	46
	10^{-3}	7	31± 3.1	6±0.4	460±103.8	88±11.5	31
17α-1.Methallyl-19-nor-testosterone	10	6	37± 3.6	8±0.8	443± 96.6	120±24.2	−41
	1	5	35± 2.5	7±0.7	594± 62.1	111±11.4	−25
17α-2.Methallyl-19-nor-testosterone	1	6	36± 3.3	7±0.6	424± 92.3	79±11.8	29
Control (castrated male-female)		59	33± 1.5	7±0.3	432± 26.7	96± 5.2	
Control (intact male-female)		38	52± 4.7	14±0.8	111± 13.5	37± 1.6	

* Mean ± S.E. (mg. per 100 gm. body weight).

ovarian growth even in a total dose up to 10 mg. Norethandrolone, norethisterone and norethynodrel exert their anti-pituitary effect, the grade of which is roughly the same. Complete inhibition of ovarian growth induced by norethandrolone or norethisterone is obtainable by a non-androgenic level of their dosage. This agrees with the results obtained in the subcutaneous assay.

2. *Uterotrophic activity*

Uterotrophic activities of six estrogens and two 19-norsteroids, norethisterone, norethynodrel are summarized in Figure 1. The order of

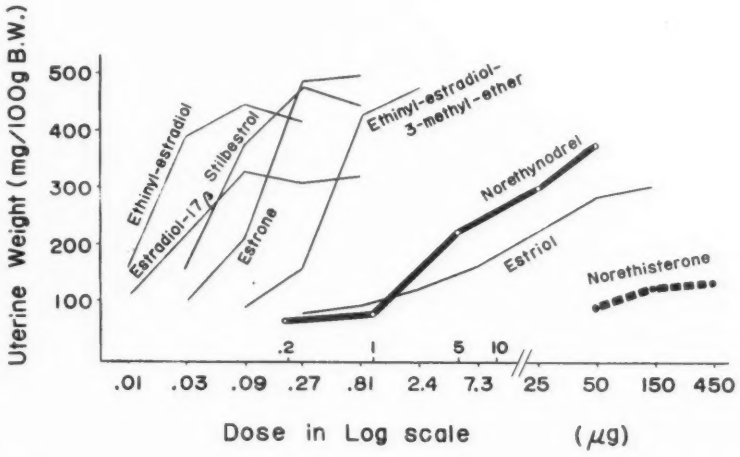

FIG. 1. Dose-response curves of various estrogens, norethynodrel and norethisterone for uterotrophic activity.

activities is ethynyl-estradiol > stilbestrol > estrone > ethynyl-estradiol-3-methylether > estriol ≒ norethynodrel ≫ norethisterone. Uterine hydration is observed at autopsy in the norethynodrel group but not in the norethisterone group.

3. *Androgenic and myotrophic activity*

The dose-response relationships of testosterone propionate on the seminal vesicles, ventral prostate and levator ani muscles are presented in Figure 2.

The data suggest that testosterone propionate in a total dose of 3.2 mg. per rat, produces the maximal response for the three targets. The responses of ventral prostate and levator ani muscles to the other compounds including norethisterone, norethynodrel and progesterone are investigated at the dosage level of 3.2 mg.

The increase ratios of the target weights against control level are plotted

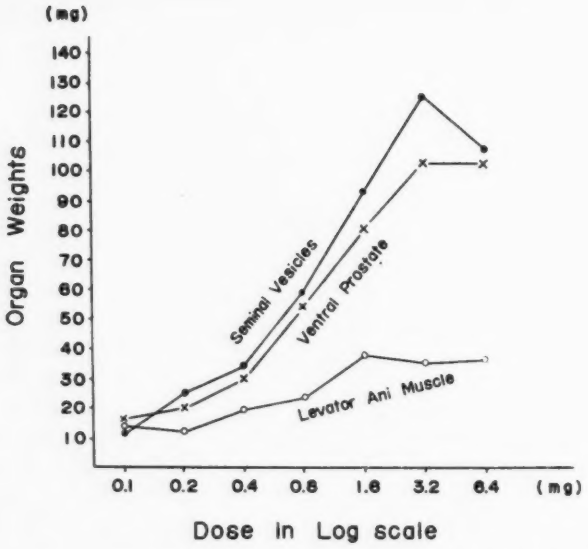

FIG. 2. Dose-response curves of testosterone propionate for androgenic and myotrophic activity.

in Figure 3, so that the characteristic potency of the compounds on the androgenic and anabolic activities are represented by each of the vectors concerning the two dimensions, the increase ratio of ventral prostate and that of levator ani muscles. The figure indicates that the three androgens, methyltestosterone, methylandrostenediol and ethynyltestosterone, the potencies of which follow this order, are qualitatively more androgenic and less anabolic than testosterone propionate. The three dihydrotestosterones and norethisterone, however, are qualitatively more anabolic and less

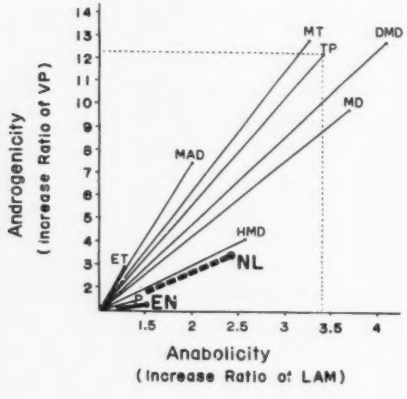

FIG. 3. Characteristic potency of various steroids on the androgenic and anabolic activity.

TP = Testosterone propionate
ET = Ethynyl-testosterone
MAD = Methyl-androstendiol
MT = Methyl-testosterone
DMD = 2,17-Dimethyl-dihydrotestosterone
MD = 2-Methyl-dihydrotestosterone
HMD = 2-Hydroxymethylene-17-methyl-dihydrotestosterone
NL = Norethisterone(17-ethynyl-19-nortestosterone)
EN = Norethynodrel(17-ethynyl-5(10)-estraenolone)

androgenic than testosterone propionate, and the order of their potencies is 2,17-dimethyl-dihydrotestosterone > 2-methyl-dihydrotestosterone > 2-hydroxymethylene-17-methyl-dihydrotestosterone ≒ norethisterone. In the order of their anabolic/androgenic ratios, the reverse is true. The activities of progesterone and norethynodrel seem to be almost negligible in this test.

4. *Progestational activity*

The data concerning the progestational activities of 19-norsteroids and progesterone derivatives in the subcutaneous test quoted from the previous paper (9) are summarized in Figure 4. The assay method is based on the

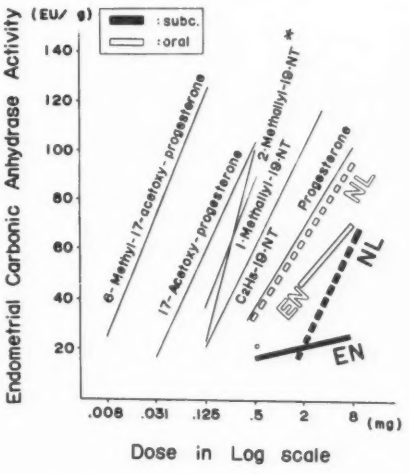

FIG. 4. Progestational activity of various steroids on the Clauberg rabbit (from the data of Miyake and Pincus, 1958).
*=Unpublished data.

measurement of endometrial carbonic anhydrase titers on the Clauberg rabbit as described in previous papers (9, 11). The order of potencies is 6-methyl-17-acetoxyprogesterone > 17-acetoxyprogesterone > 17-1.methallyl- or 17-2.methallyl-19-nortestosterone > progesterone > norethisterone > norethynodrel.

DISCUSSION

Fair reproducibility of castration hypersecretion of pituitary gonadotrophin and the assay data on a variety of steroids confirm the adaptability of the parabiotic mice method for quantitative study on the gonadotrophic secretion of the anterior pituitary.

As previously reported (6), estrogen is much more active than androgen for gonadotrophin inhibition in either male or female pituitary, and complete inhibition of gonadotrophin hypersecretion is obtainable by a non-uterotrophic level of estrogen. The data presented in this paper suggest that peripheral activity, such as uterotrophic potency, of the various

estrogens seems to be related to their anti-gonadotrophic potency.

Androgenic responses indicated by the increase of seminal vesicles and ventral prostate in the castrated partner are always accompanied by an anti-gonadotrophic response as represented by ovarian inhibition in the intact partner. However, the reverse is not true. For example, methyltestosterone, methylandrostenediol and a series of 19-nortestosterones produce complete or almost complete inhibition of ovarian growth at a dosage level with no concomitant androgenic response. Furthermore, a weak androgen, methylandrostenediol, which has approximatley 1/20 the activity of testosterone (12), exerts more potent anti-gonadotrophic activity than that of testosterone propionate. These results agree with the findings of Goldman *et al.* (1), who studied the pituitary-inhibiting, anabolic and androgenic effect of norethandrolone in parabiotic rats and emphasized that the pituitary-suppressing properties are not due to any androgenic potential the drug may have. Lerner *et al.* (3) have reported that non-androgenic dosages of 19-nortestosterone-17-benzoate can inhibit the production or release of pituitary gonadotrophin in the male rats. Most recently, Kincl and Dorfman (5) have also reported that anti-pituitary activity of certain series of synthetic androgens appears to be parallel with the myotrophic activity of the compounds rather than with their androgenic activity.

In the group of progesterone and its derivatives, anti-pituitary activity of the compounds is proportional to their progestational activity in the subcutaneous assay, but not in the oral test. The most potent of the progestational substances, 6-methyl-17-acetoxyprogesterone, is also active in subcutaneous assay for gonadotrophin inhibition, but in the oral test it does not produce any significant gonadotrophin suppression in dosages up to 10 mg. The two methallyl compounds, 17α-1.methallyl- and 17α-2.methallyl-19-nortestosterone, both of which are more potent than norethisterone or norethynodrel either subcutaneously or orally in the Clauberg assay (9, 10), surprisingly do not show any superiority in the subcutaneous anti-gonadotrophin test, and they are not active in the oral test. On the contrary, rather weak progestins such as norethisterone and norethynodrel have potent anti-pituitary activity, the oral potency of which is roughly equivalent to that of estrone. Therefore, gonadotrophin inhibition by progestational steroids cannot be the result of their progestational properties.

As suggested by the results of our experiments, estrogenicity is most likely responsible for anti-pituitary activity, but other peripheral activities of a compound are not always proportional to its anti-gonadotrophic activity. Madjerek *et al.* (13) have recently reported a new oral progestin, allylestrenol (3-deoxo-17α-allyl-19-nortestosterone), which is practically devoid of androgenic, estrogenic and gonad- and adrenal-inhibiting proper-

ties. On the basis of our previous data (6), the anti-gonadotrophic potency of estrone is approximately 10,000 times that of testosterone propionate in subcutaneous assay and 100 times in oral assay. The metabolic conversion of androgens to estrogens has been firmly established *in vivo* and *in vitro* as reviewed by Dorfman (12). Breuer *et al.* (14) and Engel *et al.* (15) have recently demonstrated that the excretion of estrogen is significantly increased by 19-nortestosterone, and ethynyl-nortestosterone is aromatized to ethynyl-estradiol in the body. Kaiser (16) has reported the increase of urinary aromatic steroids, especially the estrone fraction, by the administration of ethyl-, ethynyl- or methyl-19-nortestosterone into women. According to the data of Heller *et al.* (17) who tested with the human adult males, the urinary excretion of estrogen is increased by consecutive treatment with norethisterone or norethynodrel during a period of 43 to 48 days, but not altered by progesterone or norethandrolone similarly administered. Progesterone and desoxycorticosterone, both of which produce slight gonadotrophin inhibition, have been well recognized as versatile intermediates in the biosynthesis of steroid hormones such as corticoids, androgens and estrogens.

These facts suggest a possible explanation for the manifestation of anti-gonadotrophic effects by various steroids: that the major part of androgens. progestins or 19-norsteroids may act by themselves (or through metabolites) as biologically active substances to produce their typical peripheral effects, and that a part of those compounds may be converted into estrogenic substance, the amount of which is able to inhibit gonadotrophic secretion but is not sufficient to affect peripheral targets, so that the conversion rates determine their anti-gonadotrophic potency.

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THE USE OF PARABIOTIC MICE FOR THE STUDY OF PITUITARY GONADOTROPHIN HYPERSECRETION AND ITS INHIBITION BY STEROIDS[1]

TAMOTSU MIYAKE

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ABSTRACT

Comparative observations on a total of 551 pairs of parabiotic rats and over 1000 pairs of parabiotic mice suggest the usefulness of parabiotic mice for the study of gonadotrophin hypersecretion and its regulation by certain steroids. The advantage of the use of parabiotic mice for this study resides in the relative ease and speed of parabiotic operation, no need of littermates as long as inbred mice are used and low mortality of parabionts.

The pituitary-gonad relations observed in parabiotic mice are quite similar to those in parabiotic rats. Namely, the gonadotrophic potency of the male pituitary is larger than that of the female either in the normal or in the castrated condition, and greater amount of sexagen is required to inhibit gonadotrophin hypersecretion of the male pituitary. Estradiol is more potent than testosterone propionate in its anti-gonadotrophic activity. A lesser amount of steroid can regulate the secretion of pituitary gonadotrophin than that which stimulates the peripheral targets, especially in the female mice.

IT IS a well known fact that the parabiosis method has contributed much to the establishment of pituitary-gonad relationships; thus, in parabionts gonadotrophin hypersecretion induced by gonadectomy may be regulated by the administration of gonadal hormones. Most of the experiments on this problem have been carried out using parabiotic rats, but little has been reported on the use of parabiotic mice.

The work described here represents a comparison between parabiotic rats and mice of the gonadal hypertrophy of the intact female united with a castrated male or female and on its inhibition by two sexagens, estradiol-17β and testosterone propionate.

METHODS

Our inbred colonies of albino rats (80–100 gm., Wistar strain) and albino mice (15–17 gm., ddS strain) were used. Non-littermates were put into parabiosis by a modification of the Bunster and Meyer (1) method, in which metal wound clips were used instead of silk sutures in closing the skin incision. Intraperitoneal injection of methylhexabarbital sodium, 12.5 mg. per 100 gm. for rats and 1.6 mg. per 10 gm. for mice, was

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carried out for anesthetizing animals before and during the operation. Castration was performed on the right-handed partner at the time of parabiotic union.

Sex steroids were administered subcutaneously into the castrated partner once daily for 10 days starting on the day of parabiotic operation. Hormones administered into the rats were dissolved in sesame oil and injected in a volume of 0.1 ml. per day. The compounds injected into the mice, however, were homogenized with the suspending vehicle, which consists of an aqueous solution of sodium chloride (0.9%), polysorbate 80 (0.4%), carboxy methyl cellulose (0.5%) and benzyl alcohol (0.9%), because the daily injections of sesame oil 0.1 ml. per day resulted in poor absorption and in retardation of wound healing in the mice. Animals were autopsied on the 11th day of parabiosis.

RESULTS

Gonadal hypertrophy due to castration of a partner in parabiotic rats and mice

Intact female rats or mice were put into parabiosis with an intact or castrated partner to demonstrate the gonadotrophin hypersecretion caused by gonadectomy, which resulted in the gonadal hypertrophy of the intact female partner.

TABLE 1. GONADAL WEIGHTS OF INTACT FEMALES 10 DAYS AFTER PARABIOSIS

Species	Parabiosis with	Ovarian weight	Uterine weight
Rat	intact male	43.5 ± 5.8*	89.7 ± 17.7*
	castrated male	124.4 ± 8.4	238.6 ± 24.5
	intact female	35.0 ± 1.9	59.3 ± 5.0
	castrated female	89.0 ± 9.6	172.9 ± 25.8
Mouse	intact male	53.0 ± 3.8*	151.4 ± 28.2*
	castrated male	153.7 ± 8.2	556.0 ± 54.9
	intact female	41.7 ± 2.5	99.7 ± 6.5
	castrated female	97.3 ± 5.6	432.3 ± 41.4

* Mean ± S.E. (mg. per 100 gm. body weight).

Table 1 shows the data on the mean ovarian and uterine weight of each group 10 days after parabiosis, reflecting the gonadotrophic activity of the partner's pituitary. The pituitary gonadotrophin hypersecretion caused by gonadectomy is clearly indicated by these data. The greater gonadotrophic potency of the male pituitary is obvious in mouse parabionts as well as in rat parabionts. The increased ratios of ovarian to body weights in parabiotic mice are about the same as those in parabiotic rats.

Tables 2 and 3 show the relationship between the gonadal weight changes and the duration of parabiosis. In the castrated male-female parabionts of mice (Table 2), atrophy of the male accessory glands of the castrated partner and hypertrophy of the female gonads of intact partner progress according to the period after parabiosis, and reach a maximum 10 days after the operation. In the castrated female-female parabionts (Table 3), however, the gonadal hypertrophy in the intact partner does not attain a peak within a period of 15 days, and the uterine weight of castrated partner progressively decreases for the first 10 days and then reverses to exceed the

TABLE 2. CASTRATED MALE-FEMALE PARABIONTS OF MICE

Days after parabiosis	No. of pairs	Castrated male		Intact female	
		Seminal vesicles	Ventral prostate	Ovaries	Uterus
0	15	105 ±14*	21 ±2*	68 ± 3*	285 ±33*
4	10	71 ± 3	10 ±1	62 ± 7	165 ±18
6	10	68 ± 7	13 ±2	90 ±10	235 ±38
8	10	50 ± 5	9 ±1	134 ±12	385 ±50
10	25	49 ± 4	6 ±1	157 ± 8	556 ±55
15	10	47 ± 3	8 ±1	154 ±18	665 ±80
		Intact male		Intact female	
10	25	138 ±12	21 ±2	53 ± 4	151 ±28

* Mean ±S.E. (mg. per 100 gm. body weight).

non-castrated level. This suggests that the excessive amount of estrogen produced by the stimulated ovaries of the intact partner recirculates into the castrated partner and reaches a concentration sufficient to produce uterine stimulation.

The inhibitory effect of steroids on gonadotrophin hypersecretion in parabiotic rats and mice

The gonadotrophin inhibiting effects of estradiol-17β and testosterone propionate were investigated in terms of dose-response curves for ovarian weights of both parabiotic rats and mice. Hormones were administered subcutaneously once daily for 10 days starting on the day of parabiosis into the castrated partner. The results are presented in Tables 4, 5, 6 and 7.

The data on both parabiotic rats and mice indicate that estradiol is much more potent in gonadotrophin inhibition than testosterone propionate for either the male or female pituitary, and the female pituitary is more sensitive than the male pituitary to both sexagens, estradiol and testosterone propionate. The activities of both sexagens stimulating the peripheral targets are indicated by the uterus or ventral prostate weight of the castrated partner. The data indicate that the hypophysis is more sensitive

TABLE 3. CASTRATED FEMALE-FEMALE PARABIONTS OF MICE

Days after parabiosis	No. of pairs	Castrated female		Intact female	
		Uterus		Ovaries	Uterus
0	15			69 ± 8*	285 ±33*
4	10	107 ±10*		55 ± 3	146 ±14
6	10	98 ± 7		69 ± 6	190 ±37
8	10	82 ± 5		96 ±11	333 ±52
10	25	89 ± 3		97 ± 6	432 ±41
15	10	121 ±14		141 ±14	547 ±58
		Intact female		Intact female	
10	25	Ovaries 47 ±2	Uterus 102 ±8	42 ±3	100 ±7

* Mean ±S.E. (mg. per 100 gm. body weight).

TABLE 4. GONADOTROPHIN INHIBITING ACTIVITY OF ESTRADIOL IN PARABIOTIC RATS

A. Castrated Male-Female

Total dose (μg)	No. of pairs	Castrated		Intact	
		Seminal vesicles	Ventral prostate	Ovaries	Uterus
0	10	24 ± 2*	11 ± 1*	132 ± 13*	247 ± 18*
0.025	10	24 ± 3	10 ± 1	119 ± 7	207 ± 21
0.05	10	25 ± 3	10 ± 1	130 ± 8	220 ± 18
0.1	10	19 ± 2	8 ± 1	102 ± 8	161 ± 16
0.2	10	30 ± 1	13 ± 1	92 ± 9	199 ± 30
0.4	10	10 ± 1	10 ± 1	85 ± 14	181 ± 20

B. Castrated Female-Female

Total dose (μg)	No. of pairs	Castrated	Intact	
		Uterus	Ovaries	Uterus
0	10	83 ± 7*	104 ± 4*	225 ± 16*
0.005	10	69 ± 6	108 ± 8	184 ± 21
0.01	10	58 ± 5	98 ± 11	186 ± 18
0.025	10	76 ± 10	68 ± 9	145 ± 7
0.05	10	68 ± 5	54 ± 6	101 ± 17
0.1	10	86 ± 6	48 ± 4	111 ± 13
0.4	10	116 ± 11	39 ± 5	85 ± 17
0.8	10	209 ± 17	49 ± 6	88 ± 12

* Mean ± S.E. (mg. per 100 gm. body weight).

TABLE 5. GONADOTROPHIN INHIBITING ACTIVITY OF TESTOSTERONE PROPIONATE IN PARABIOTIC RATS

A. Castrated Male-Female

Total dose (μg)	No. of pairs	Castrated		Intact	
		Seminal vesicles	Ventral prostate	Ovaries	Uterus
0	10	24 ± 2*	11 ± 1*	132 ± 13*	247 ± 18*
25	10	21 ± 7	11 ± 1	139 ± 13	199 ± 17
50	10	21 ± 2	15 ± 1	94 ± 11	170 ± 27
100	10	43 ± 6	25 ± 3	56 ± 10	89 ± 18
200	10	91 ± 8	48 ± 6	44 ± 7	73 ± 13

B. Castrated Female-Female

Total dose (μg)	No. of pairs	Castrated	Intact	
		Uterus	Ovaries	Uterus
0	10	83 ± 7*	104 ± 4*	225 ± 16*
0.25	20	69 ± 7	100 ± 4	230 ± 21
0.5	20	68 ± 17	105 ± 17	187 ± 13
1	10	63 ± 6	108 ± 8	167 ± 10
2.5	10	84 ± 6	65 ± 9	145 ± 28
6.25	11	61 ± 6	42 ± 6	70 ± 9

* Mean ± S.E. (mg. per 100 gm body weight).

TABLE 6. GONADOTROPHIN INHIBITING ACTIVITY OF ESTRADIOL IN PARABIOTIC MICE

A. Castrated Male-female

Total dose (μg)	No. of pairs	Castrated		Intact	
		Seminal vesicles	Ventral prostate	Ovaries	Uterus
0	10	48 ± 7*	8 ± 1*	145 ± 16*	558 ± 23*
0.01	12	47 ± 2	7 ± 1	150 ± 14	575 ± 50
0.02	10	50 ± 4	7 ± 1	155 ± 18	540 ± 46
0.04	10	62 ± 5	11 ± 1	117 ± 12	374 ± 42
0.08	10	47 ± 4	9 ± 3	104 ± 13	351 ± 40
0.16	10	70 ± 11	11 ± 1	69 ± 6	191 ± 31
0.4	10	96 ± 9	12 ± 1	79 ± 4	260 ± 50

B. Castrated Female-Female

Total dose (μg)	No. of pairs	Castrated	Intact	
		Uterus	Ovaries	Uterus
0	10	76 ± 10*	111 ± 10*	419 ± 53*
0.00001	10	67 ± 3	100 ± 8	382 ± 64
0.000025	10	79 ± 8	94 ± 7	449 ± 57
0.00005	10	89 ± 6	61 ± 6	225 ± 49
0.000125	10	93 ± 10	59 ± 6	135 ± 26
0.00025	10	97 ± 4	73 ± 9	233 ± 55
0.0005	10	93 ± 7	65 ± 6	149 ± 13
0.00125	10	98 ± 10	60 ± 6	179 ± 45
0.0025	10	107 ± 8	54 ± 4	161 ± 44
0.005	10	92 ± 6	47 ± 3	127 ± 26
0.01	10	88 ± 6	61 ± 8	119 ± 32
0.02	10	129 ± 6	48 ± 2	77 ± 7
0.04	10	133 ± 10	56 ± 3	88 ± 5
0.08	10	177 ± 15	51 ± 2	87 ± 8
0.16	10	347 ± 40	52 ± 4	102 ± 9
0.4	10	439 ± 23	55 ± 5	116 ± 10
0.8	10	449 ± 15	53 ± 5	115 ± 10

* Mean ± S.E. (mg. per 100 gm. body weight).

than peripheral targets to sex steroids, especially in the female.

These relations observed are quite similar in parabiotic rats and mice as shown in the diagram of Figure 1.

DISCUSSION

Gonadectomy induces gonadotrophic hypersecretion in rats and this hypersecretion can be regulated by administration of gonadal hormone, (2). Various facts demonstrated in the rats, such as the greater gonadotrophic potency of the male hypophysis than the female (3), the larger dose of sexagens required to inhibit the male pituitary than the female (4, 5) and the higher sensitivity of pituitary to sexagens than that of gonads (5), are also shown in our studies of parabiotic mice.

The only disadvantage of the parabiotic mice method is that mice are too small to stand the multiple injections of oil used as a hormone vehicle. Accordingly, the compounds should be administered in suspension to the mouse parabionts, but the sensitivity in the mouse test is not inferior to that of the rat test in which oil may be used as a vehicle.

TABLE 7. GONADOTROPHIN INHIBITING ACTIVITY OF TESTOSTERONE PROPIONATE IN PARABIOTIC MICE

A. Castrated Male-Female

Total dose (μg)	No. of pairs	Castrated		Intact	
		Seminal vesicles	Ventral prostate	Ovaries	Uterus
0	10	48 ± 7*	8 ± 1*	145 ± 16*	558 ± 23*
25	20	65 ± 6	10 ± 1	129 ± 7	524 ± 47
50	10	67 ± 7	17 ± 2	107 ± 11	414 ± 73
100	20	87 ± 10	18 ± 1	85 ± 6	279 ± 32
200	20	125 ± 5	20 ± 1	77 ± 6	242 ± 38
400	20	229 ± 17	28 ± 2	59 ± 5	139 ± 12

B. Castrated Female-Female

Total dose (μg)	No. of pairs	Castrated	Intact	
		Uterus	Ovaries	Uterus
0	10	76 ± 10*	111 ± 10*	419 ± 53*
0.1	10	90 ± 6	113 ± 5	388 ± 39
0.2	10	83 ± 5	89 ± 5	503 ± 22
0.4	10	88 ± 6	75 ± 7	368 ± 73
0.625	20	109 ± 4	63 ± 5	161 ± 24
1.25	10	111 ± 8	53 ± 3	160 ± 34
2.5	10	119 ± 11	54 ± 3	117 ± 23
5.0	10	89 ± 6	44 ± 6	96 ± 10

* Mean ± S.E. (mg. per 100 gm. body weight).

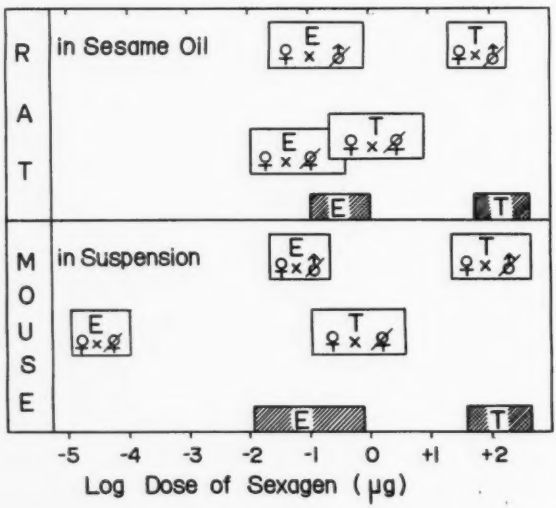

FIG. 1. Comparison of pituitary-gonad relations between parabiotic rats and mice. Clear area: dosage range of sexagen for inhibiting gonadotrophic hypersecretion. Shadowed area: dosage range of sexagen for stimulating peripheral targets. E: estradiol-17β. T: testosterone propionate.

TABLE 8. MORTALITY OF PARABIOTIC MICE

Date of experiments	No. of pairs	No. of dead	Mortality (%)
April, 1959	198	8	4
Sept.–Nov., 1959	580	70	12
Jan.–Mar., 1960	252	28	11
Total	1030	106	10

High mortality of parabiotic rats has been pointed out by Finerty and Panos (6) who reported that mortality of rats from parabiosis intoxication was 64% in non-littermates and 50% in littermate pairs. Our experience with rat parabiosis agreed with theirs, although our mortality rate seemed to be smaller than theirs. In regard to mouse parabiosis, however, extremely low mortality (approx. 10%) has been obtained even in non-littermate pairs (Table 8).

About 17% of control pairs of intact-castrated parabiotic mice failed to show any gonadal stimulation, as shown in Table 9. This figure is slightly

TABLE 9. PERCENTAGE FAILURE OF GONADOTROPHIC HYPERSECRETION IN CASTRATED MALE-FEMALE PARABIOTIC MICE

Date of experiment	No. of control pairs	Pairs failed to respond*	Failure (%)
April, 1959	30	5	17
Sept.–Nov., 1959	29	2	7
Jan.–Mar., 1960	116	21	18
Total	175	30	17

* Number of pairs which failed to show any gonadal stimulation in the intact partner.

larger than that (11 to 13%) reported by Hertz and Meyer (4) or by Biddulph *et al.* (7) on littermate rats.

Elimination of the necessity for the use of littermates and the low mortality of parabionts offer great advantages in the use of parabiotic mice for the study of pituitary-gonad relations, and therefore for the assay of anti-gonadotrophic activity of various steroids.

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STUDIES ON THE SERIAL EXTRACTION OF PITUITARY PROTEINS[1]

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ABSTRACT

Pituitary glands of beef, sheep and pig origin were successively extracted with water at pH 5.5, M/10 $(NH_4)_2SO_4$ at pH 4.0, M/4 $(NH_4)_2SO_4$ at either pH 5.5 or 7.5, and finally with 60 percent ethanol at pH 9.8 to 10. The extracts were bioassayed for the anterior pituitary hormones and for the proteinases I and II. With certain exceptions, the first extract contained mainly FSH and proteinase I; the second, LH and TSH, the third, GH and proteinase II; and the fourth, prolactin. FSH and GH were further purified to establish the amenability of these extracts to fractionation. Growth hormone isolated from beef pituitary extracts prepared at pH 5.5 appears to be a mixture of two biologically active proteins: one with alanyl and phenylalanyl NH_2-terminal groups and another with methionyl and alanyl NH_2-terminal groups. Small amounts of NH_2-terminal serine and glutamic acid were also present. By varying the conditions of extraction appropriately, the amounts of the two types of hormones could be varied inversely. On prolonged extraction at pH 5.5 and 20°, the growth hormone with NH_2-terminal methionine had almost completely replaced that with NH_2-terminal phenylalanine. It is concluded that methionyl growth hormone is an artifact of the isolation procedure and is derived from the phenylalanyl hormone by the hydrolytic action of previously undescribed proteinases, optimally active at pH 5 to 6.5. On the basis of sedimentation, NH_2-terminal, and methionine analyses, the methionyl growth hormone does not differ significantly in molecular weight from conventional growth hormone. Furthermore, it is inferred that of the seven bonds in the hormone containing methionine only the one nearest the NH_2-terminus in the phenylalanyl chain is cleaved. Two new proteolytic enzymes were found in the pituitary. One hydrolyzes the Ser·Met- bond in Ser·Met·Glu (NH_2) and CH_3CO·Ser·Tyr·Ser·Met·Glu(NH_2) and has a hydrolytic optimum at pH 4.5 to 5. The other readily hydrolyzes the -Met·Glu (NH_2) bond in the tripeptide but has relatively little action on the pentapeptide. Its optimum is at pH 6 to 7.0. Sheep extracts yield growth hormone having the usual phenylalanyl and alanyl NH_2-terminal groups. Since sheep extracts obtained at pH 5.5 also contain proteinases capable of hydrolyzing methionyl peptides, the absence of a methionyl NH_2-terminal group in the isolated sheep growth hormone is interpreted to mean that peptide bonds susceptible to the pro-

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teinases are absent or inaccessible. Bovine growth hormone isolated from M/4 $(NH_4)_2SO_4$ extracts prepared at pH 5.5 was characterized by a higher electrophoretic mobility than that isolated from alkaline extracts. Treatment with mildly alkaline solutions produced a partial conversion to a slower electrophoretic component whose mobility approximated that of growth hormone prepared from alkaline extracts.

IN a previous investigation (1) a scheme was described for the separation and partial purification of the following hormones and enzymes from sheep pituitary glands: follicle-stimulating hormone, luteinizing hormone, thyrotropic hormone, growth hormone[4] and the proteinases I and II (2). Since the method of extraction was not selective, all of these proteins were obtained together in a single extract from which the different hormones and enzymes were subsequently separated from one another by salt or ethanol fractionation and by chromatography.

In the present investigations, attention has been devoted toward separating the above proteins during the initial extraction of pituitary glands by varying the conditions of pH and ionic strength. This report describes the distribution of hormones and enzymes in extracts obtained by successively treating pituitaries of beef, sheep, and pig origin with the following solvents: distilled water at pH 5.5, M/10 $(NH_4)_2SO_4$ at pH 4.0 and M/4 $(NH_4)_2SO_4$ at pH 5.5 or 7.5. Growth hormone isolated from beef and sheep extracts obtained with the latter solvent system was studied with respect to electrophoretic properties and NH_2-terminal groups. The beef growth hormone was found to contain methionine as one of the NH_2-terminal groups in addition to phenylalanine and alanine as reported by Li and Ash (3) for growth hormone isolated from alkaline extracts. The latter portion of this report is concerned with heretofore undescribed proteinases, optimally active at pH 5.0 to 6.5, which appear to be responsible for the formation of beef growth hormone containing NH_2-terminal methionine.

METHODS

All extractions and fractionations were performed at 5°. With the exception of LH, bioassay for hormone and proteinase activities was performed by the methods described in a previous publication (1). LH was assayed by the ovarian hyperemia method (4) wherein the degree of hyperemia of the ovaries is quantitatively estimated from their content of previously injected radio-iodinated serum albumin. Occasionally, the ventral prostate method of Greep *et al.* (5), with hypophysectomized rats of the Sprague-Dawley strain, was used to confirm the results of hyperemia method. In all assays potencies were estimated from log dose-response curves obtained from a series of graded doses of both the test and the standard preparation. The standards against which potencies of the fractions were measured were Armour LH #227-80 (1 unit per mg.), Armour FSH #264-151-X (1 unit per mg.), U.S.P. beef growth hormone (1 unit per mg.), U.S.P. thyrotropin reference standard, and N.I.H. prolactin[5] (15 I.U. units per mg.). Prolactin potency was measured by the crop sac weight method (6).

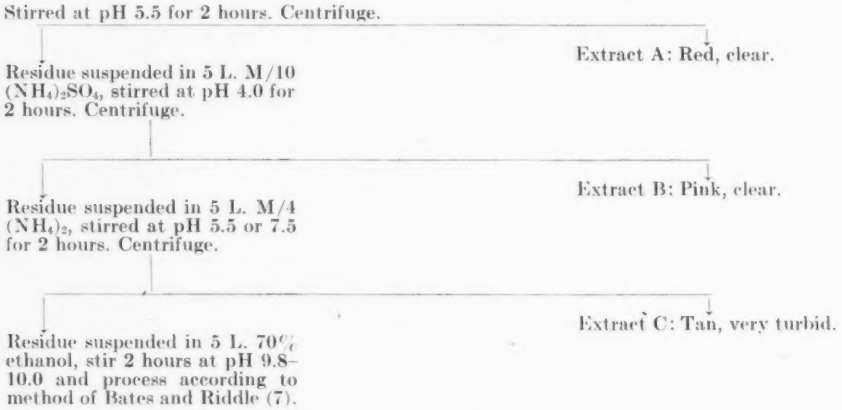
[4] Abbreviated as FSH, LH, TSH, and GH, respectively.

[5] Gift of the Endocrinology Study Section, National Institutes of Health.

PROCEDURES AND RESULTS

Extraction with Water at pH 5.5: Frozen whole pituitaries of either beef, sheep or pig origin were warmed to about −5° at which temperature they could be conveniently ground in an electrically powered meat grinder. The glands were passed once through the grinder equipped with a plate having $\frac{1}{4}$ inch holes and then twice through a plate with 1/16 inch holes. This procedure which was performed in a 5° cold room assured fine grinding and facilitated efficient extraction. The glands were then thoroughly mixed with 5 liters of distilled water per kg. of glands and adjusted to pH 5.5 with 2 M HCl.

The suspension was then extracted according to the following diagram:


Stirred at pH 5.5 for 2 hours. Centrifuge.
Extract A: Red, clear.
Residue suspended in 5 L. M/10 $(NH_4)_2SO_4$, stirred at pH 4.0 for 2 hours. Centrifuge.
Extract B: Pink, clear.
Residue suspended in 5 L. M/4 $(NH_4)_2$, stirred at pH 5.5 or 7.5 for 2 hours. Centrifuge.
Extract C: Tan, very turbid.
Residue suspended in 5 L. 70% ethanol, stir 2 hours at pH 9.8–10.0 and process according to method of Bates and Riddle (7).

The yields of the dialyzed and lyophilized extracts, specific and total activities obtained from the pituitaries of each species is summarized in Table 1. From this data it can be seen that beef extract A removes almost 50% of the soluble, but hormonally inert, protein so that the succeeding extracts are considerably enriched in TSH, LH, and GH. Thus, TSH and LH in beef extract B are 4 to 5 times more concentrated than in alkaline extracts (8–12) and the GH in extract C is about 20% of the potency of highly purified growth hormone. Sheep extract A contains nearly 90% of the glandular FSH and hence would appear to be a suitable starting material for the isolation of FSH. As in the case of the beef hormone, sheep TSH and LH are concentrated into extract B and GH into extract C. Pig glands yield an extract A, which differs from corresponding sheep and beef extracts in that it contains not only the major portion of the FSH but also about 60% of the glandular LH. The LH content can be further increased in this extract at the expense of extract B by re-suspending the residue in $\frac{1}{2}$ the original volume of water, and re-extracting at pH 5.5 for 1 hr. The combined solutions contain 90 and 76% of the glandular FSH and LH, respectively. Only negligible increases in the amount of other hormones is incurred by the

TABLE I. DISTRIBUTION OF HORMONE AND PROTEINASE ACTIVITIES AND YIELDS IN SUCCESSIVE EXTRACTS OF PITUITARY GLANDS*

Extract†	Yield, gm./kg.	FSH units per		LH units per		TSH units per		GH units per		PRO I units per		PRO II units per	
		mg.	kg.	mg.	kg.	mg.	kg.	mg.	kg.	mg.N	kg.	mg.N	kg.
A (beef)	35.0 (47.5%)	0	0	0	0	0.016	560 (16%)	0.012	420 (7%)	5.4	30,240 (67%)	0.04	226 (2%)
B (beef)	10.0 (13.5%)	0	0	0.04	400 (65%)	0.20	2,000 (58%)	0.04	400 (6%)	4.0	6,400 (14%)	0.06	96 (1%)
C (beef)	26.5 (35.9%)	0	0	0.008	212 (35%)	0.034	900 (26%)	0.2	5,300 (85%)	2.0	8,500 (19%)	2.2	9,340 (92%)
C-1 (beef)	2.3 (3.1%)	0	0	—	—	—	—	0.04	90 (2%)	0.2	80	1.4	515 (5%)
Total	73.8		0		612		3,460		6,210		45,220		10,177
A (sheep)	34.4 (40%)	0.043	1,480 (86%)	0.011	380 (31%)	0	0	0.015	515 (10%)	5.6	30,800 (60%)	0.36	1,980 (15%)
B (sheep)	7.4 (8.6%)	0.033	244 (14%)	0.085	630 (51%)	0.16	1,280 (76%)	0.027	200 (4%)	4.8	5,660 (11%)	0	0
C (sheep)	28.2 (32.8%)	0	0	0.008	226 (18%)	0.014	400 (24%)	0.14	3,950 (78%)	3.2	14,400 (28%)	1.68	7,560 (57%)
C-1 (sheep)	16.04 (18.6%)	0	0	—	—	—	—	0.027	435 (8%)	0.2	520 (1%)	1.4	3,600 (27.4%)
Total	86.04		1,724		1,236		1,680		5,100		51,380		13,140
A (pig)	41.9 (49.5)	0.18	7,540 (80%)	0.019	795 (59%)	0.024	402 (10%)	0	0	3.8	25,400 (42%)	0.4	2,680 (35.5%)
B (pig)	11.9 (14.1%)	0.12	1,430 (15%)	0.033	394 (29%)	0.25	2,975 (76%)	0	0	4.8	9,140 (15%)	0	0
C (pig)	23.7 (28.0%)	0.02	475 (5%)	0.007	166 (12%)	0.024	569 (14%)	0.4	9,480 (87%)	6.8	25,800 (43%)	0.8	3,040 (40.3%)
C-1 (pig)	7.15 (8.4%)	0	0	—	—	—	—	0.2	1,400 (13%)	0	0	1.6	1,830 (24.2%)
Total	84.65		9,445		1,355		3,946		10,880		60,340		7,550

* Yields are calculated per kg. of fresh glands. All values shown are for a single extraction at each pH; see text for effects of a second extraction. Units per mg. refers to the specific activity of the lyophilized extract; units per kg. refers to total units of activity in an extract obtained from one kg. of glands.

† Extract A, pH 5.5 water extract; extract B, pH 4.0 M/10$(NH_4)_2SO_4$; extract C, pH 7.5 M/4$(NH_4)_2SO_4$; C-1, pH 5.5 precipitate from extract C.

re-extraction. As with sheep and beef extracts, TSH and GH are concentrated into extracts B and C, respectively.

Each of the beef and sheep extracts shown in Table 1 were assayed for corticotropic activity in hypophysectomized rats by the ascorbic acid depletion test. In all fractions the content of corticotropin amounted to not more than a few U.S.P. milliunits per mg. with the exception of sheep extract B which had an activity equivalent to 20 U.S.P. milliunits per mg.

In assays of extracts A, B, and C for prolactin, no increase in crop sac weight or even a mucosal stimulation was evident as 10 mg. total doses. A little prolactin activity, not more than 0.6 I.U. per mg., was detected in sheep fraction C-1. In view of the possibility that prolactin may have been destroyed by the pituitary proteolytic enzymes during the extractions between pH 4.0 and 7.5 at 5° C, the residue from extract C was utilized for extraction of prolactin by the method of Bates and Riddle (7). The yield of protein was 19.3 and 10.9 g. from sheep and beef residues. The potency of the sheep and beef preparations was 4 and 3 I.U., respectively. Hence the content of prolactin in the residues was calculated to be 2.6 and 1.1 g. per kg. of sheep and beef glands, respectively, assuming pure prolactin to contain 30 I.U. per mg.

Extractability of GH and Proteinase II: Whereas extract A and B from each species of pituitary were transparent solutions after centrifugation for 1 hour at 1000–2000$\times g$., extract C was extremely turbid and difficult to decant from the slimy sediment. The refractory behavior of the pH 7.5, M/4 $(NH_4)_2SO_4$ extract with regard to clarification by centrifuging prompted an investigation of other possible methods of extraction. Aliquots of the residue obtained by extraction with M/10 $(NH_4)_2SO_4$ at pH 4.0 were extracted with the solvents shown in Table 2. Two hundred grams of residue, equivalent to 276 gm. of fresh glands, were resuspended in 1 liter of water or M/4 $(NH_4)_2SO_4$ and extracted overnight at the pH values indicated. The suspensions were adjusted to pH 3 and 4 with 2M HCl and to pH 9.0 or higher with a 10% CaO slurry or octylamine. After centrifugation the supernatant solutions were neutralized, dialyzed free of salt and lyophilized. The yield, bioassay data and the appearance of the supernatant solutions after centrifugation are outlined in Table 2. It is evident that the optimum conditions for obtaining maximal yields of both GH and proteinase II entail extraction with M/4 $(NH_4)_2SO_4$ at 5.5 or 7.5. Although nearly clear and colorless supernatant solutions and compact residues were obtained with distilled water at pH 3 and 4, the yields of GH and proteinase II were negligible. Very satisfactory clarification of the extracts was obtained with CaO and octylamine at pH 9; however, low yields of the two activities were encountered. Hence, extraction at pH 7.5 or 5.5 appears to be the most efficient procedure despite clarification difficulties which, however, are less pronounced at pH 5.5 than at pH 7.5.

Concentration of FSH from Sheep Extract A: Previous studies (1) on the

TABLE 2. EXTRACTABILITY OF GH AND PROTEINASE II FROM pH 4.0, M/10 $(NH_4)_2SO_4$ RESIDUES

Conditions of extracts	Appearance of extract	Yield, gm./kg. glands	GH		PRO II	
			Units/mg. extract	gm./kg. glands	Units/mg. extract N	Units/kg. glands
H_2O, pH 3.0	Clear	3.37	0.03	0.03	0.3	160
H_2O, pH 4.0	Clear	1.56	0.03	0.01	0	0
M/4 $(NH_4)_2SO_4$, pH 5.5	Turbid	15.35	0.4	6.2	2.4	5,880
M/4 $(NH_4)_2SO_4$, pH 7.5	Very turbid	29.6	0.27	8.0	2.2	10,400
$Ca(OH)_2$, pH 9.0	Clear	8.07	0.31	2.5	3.0	3,880
Aqueous octylamine, pH 9.0	Clear	4.64	0.36	1.6	0.2	150
$Ca(OH)_2$, pH 10.0	Very turbid	8.56	0.16	1.4	1.6	2,200
$Ca(OH)_2$, 0.2 M KCl, pH 10.0	Extremely turbid	25.1	0.16	4.0	1.8	7,240
$Ca(OH)_2$, pH 11.0	Very turbid	10.0	0.35	3.5	1.6	2,560

purification of sheep FSH have shown that the amenability of this hormone to fractionation is dependent on the pH employed for preparation of the extract. Extract A was therefore fractionated with $(NH_4)_2SO_4$ to determine whether the separation and concentration of FSH could easily be accomplished. The pH 5.5 distilled water extract from 1 kg. of sheep pituitaries, prepared as described above, was taken to 2.0 M $(NH_4)_2SO_4$ at pH 5.5. The lightly turbid solution was then adjested to pH 4.0 with 2 M HCl and the copious dark brown precipitate which formed was removed by centrifugation. To the supernatant solution, $(NH_4)_2SO_4$ was added to 3 M to precipitate the FSH which was then re-suspended in a minimal quantity of water and dialyzed free of salt. Further inert impurities were precipitated by adjusting a 5 to 6% solution of the dialyzed FSH to pH 5.5. The precipitate and the supernatant solution containing the FSH were neutralized and recovered by lyophilization. The yields and potencies of the different fractions are shown in Table 3. By this procedure a two-fold concentration of FSH activity was achieved with no loss of activity. The FSH concentrate can then be purified further by chromatography on DEAE-cellulose (1, 13) followed by zone electrophoresis on starch as outlined in a previous report (1). The final product has a potency approximately 50 times the Armour FSH Standard 264-151-X.

Fractionation of Extract C for GH: It is apparent from Table 1 that

TABLE 3. FRACTIONATION OF EXTRACT A FOR FSH AND PROTEINASE I BY $(NH_4)_2SO_4$ AND BY ETHANOL

Fraction	Yield, gm./kg. glands	FSH		Proteinase I	
		Units/mg. fraction	Units/kg. glands	Units/mg. N in fraction	Units/kg. glands
2M $(NH_4)_2SO_4$, pH 4.0 ppt.	18.5	0	0	9.6	28,400
3M $(NH_4)_2SO_4$, ppt.	1.0	1.0	1000	3.4	540
pH 5.5 ppt.	0.38	0	0	—	—
pH 5.5 supernatant	0.57	2.0	1140	—	—

growth hormone is the only major hormonal activity present in extract C. It would therefore be expected that the preparation of purified growth hormone practically free from its usual contaminants, TSH and LH, should be relatively straight-forward. However, it should be noted that growth hormone has been exposed to possible action by proteinase I during extraction at pH 4.0 and to proteinase II during extraction at pH 7.5. As a result of some action by these enzymes growth hormone may have been altered in solubility properties such that it no longer can be satisfactorily purified by established procedures designed for alkaline extracts (14, 15). For the purpose of establishing the amenability of extract C to fractionation for growth hormone and also to determine the recovery of the purified hormone, extract C from beef and sheep glands was fractionated for growth hormone by the following procedure. The pH 5.5, M/4 $(NH_4)_2SO_4$ extract of either beef or sheep pituitaries was adjusted to pH 7.5 with 1 M NaOH and $(NH_4)_2SO_4$ was added to final concentration of 1.0 M. The resulting precipitate was discarded and the opalescent supernatant solution was brought to 1.8 M $(NH_4)_2SO_4$. The precipitate was dialyzed until salt-free and then re-suspended in approximately $\frac{1}{2}$ of the original extraction volume so as to give a 1.0% protein solution. The pH was then adjusted to 4.0 and the highly turbid suspension was extracted for 30 minutes with continuous stirring. The insoluble residue was removed by centrifugation and the slightly turbid supernatant solution was carefully adjusted to pH 5.4 to 5.5 with 1 M NaOH. The precipitate was removed and the GH in the supernatant solution was recovered by precipitation with 1.8 M $(NH_4)_2SO_4$ at pH 5.5 to 7.5. Alternatively, to assure a minimal amount of contamination with TSH, the final precipitation may be performed at 1.25 M with due precaution to maintain a pH of 4.0 during the salt addition (15). This precipitate was resuspended in water, neutralized before and after dialysis, and recovered by lyophilization. The yield of hormone by either procedure was 4.7 g. and 4.3 g. per kg. of fresh whole beef and sheep pituitaries, respectively. Analysis of the products by bioassay showed a potency of 0.9 to 1.2 times that of the U.S.P. growth hormone. Both preparations contained less than 0.01 U.S.P. units of TSH per mg. and not more than 0.1% of LH relative to Armour LH-#227-80 or NIH-LH-SI. The recovery of hormone activity relative to the content in extract C was calculated to be 71 and 87 percent from beef and sheep glands, respectively. Hence, with regard to potency and freedom from the usual hormonal contaminants, these preparations of growth hormone compare favorably with those prepared by other methods (14, 15, 16). Moreover, the yield is considerably greater than heretofore reported.

Electrophoresis of Beef Growth Hormone Prepared from Extract C: Electrophoretic analysis of the 1.8 M $(NH_4)_2SO_4$ precipitate of beef growth hormone was performed at pH 4.0 in 0.1 M $NaC_2H_3O_2$–0.45 M $HC_2H_3O_2$ buffer and at pH 9.0 in 0.1 M sodium barbital-0.007 M HCl buffer. The patterns obtained in the pH 4.0 buffer showed the presence of two components

whose respective mobilities were $+6.3\times10^{-5}$ and 4.6×10^{-5} $cm^2 \cdot sec^{-1} volt^{-1}$. At pH 9.0, only a single component with a mobility of -2.2×10^{-5} $cm^2 \cdot sec^{-1} volt^{-1}$ was evident. In six different preparations, the percentage of the two components at pH 4.0 varied between 72 and 60% fast component and 28 to 40% slow component as computed from the areas in the descending boundaries. In contrast to these results, when beef growth hormone was isolated from *alkaline* extracts according to the method of Li (14) only one component was evident on electrophoresis in pH 4.0 or 9.0 buffers, the respective mobilities being 4.6 and -2.2×10^{-5} $cm^2 \cdot sec^{-1} volt^{-1}$. It should be noted that the mobility at pH 4.0 corresponds exactly with that of the slow component in growth hormone prepared from extract C. Notwithstanding the grossly different mobility of the major component of growth hormone from extract C, both this and the Li preparations were equipotent with the U.S.P. growth hormone. Thus, repeated multiple dose assays on both preparations revealed that the potencies range from 0.9 to 1.2 times the U.S.P. hormone and that the mean potencies were not significantly different at 95% confidence limits.

While attempting to separate the fast and slow electrophoretic components by conventional methods such as fractionation with ethanol or isoelectric preparation, it was observed that a conversion of the fast to the slow component occurred, particularly, in mildly alkaline solutions. For the purpose of relating the change to pH, the hormone was exposed to solutions ranging from pH 5.3 to 10 for roughly comparable periods of time after which the hormone was analyzed electrophoretically. The results are shown in Table 4. The conversion proved to be most rapid in alkaline solutions with little or no change occurring below pH 6.0. The conversion of fast to slow component appeared to cease when the latter reached an upper limit of 70%. With regard to biological activity, none of the foregoing treatments resulted in a loss of growth promoting potency. Hence, in view of the constancy of biological activity during these electrophoretic conversions it may be concluded that both components have the same degree of growth promoting activity.

TABLE 4. ELECTROPHORETIC ANALYSES OF GROWTH HORMONE AFTER EXPOSURE TO SOLUTIONS AT DIFFERENT pH VALUES

Solvent	Hours of treatment	% component[1]	
		Fast	Slow
Untreated	0	60	40
M/10 $NaC_2H_3O_2$—$HC_2H_3O_2$, pH 5.3	5	60	40
M/10 $NaC_2H_3O_2$—$HC_2H_3O_2$, pH 5.3	11	60	40
M/10 NaH_2PO_4—NaOH, pH 6.0	16	58	42
.05 M Tris-HCl, pH 7.5	11	36	64
2% $NaHCO_3$, pH 8.2	18.5	34	66
2% $NaHCO_3$, pH 8.2	28	33	67
M/10 Glycine-NaOH, pH 10	21	28	72

[1] Electrophoresis performed in 0.1 M $NaC_2H_3O_2$–0.45 M $HC_2H_3O_2$ buffer, pH 4.0, $\Gamma/2 = 0.1$. Areas measured on descending limb. All solutions were 1% in protein concentration.

The results shown in Table 4 suggested that if the isolation of beef growth hormone was performed in solutions below pH 6.0, the transformation might be avoided. This did not prove to be correct, since even though extract C was prepared at pH 5.5 and fractionated as described above without ever exceeding pH 5.5, the resulting growth hormone still contained the same proportions of fast and slow components. Hence, whereas the *purified* hormone does not undergo the electrophoretic conversions when maintained below pH 6.0, the hormone present in crude extracts or fractions appears to do so even in the acid pH range.

Although the growth hormone prepared from beef extract C is as stable biologically in mildly alkaline solutions as the hormone prepared by other methods (15, 17), the electrophoretic transformations observed in such solutions are quite different. Thus, Li and Papkoff (17) observed that exposure of the hormone to pH 9.4 buffer results in the formation of a fast moving component on electrophoresis of the product at pH 4.0, whereas Ellis *et al.* (18) were unable to duplicate these observations with their hormone preparation. The present observations constitute still another type of electrophoretic transformation in alkaline solution, namely, conversion of a fast component to a slower one which is the reverse of that described by Li. Since the 3 sets of observations were made on growth hormone prepared by different methods, it is reasonable to assume that it is the method of preparation which is responsible for the differing electrophoretic properties of each preparation. Judging from the electrophoretic conversions incurred in solutions as mild at pH 7.5, it appears that growth hormone isolated from pH 5.5 extracts is a much more metastable protein than that isolated from alkaline extracts. Moreover, the conversion to an electrophoretic form with a mobility apparently identical with that of the hormone isolated from alkaline extracts strongly suggests that the fast component of growth hormone isolated from pH 5.5 extracts may well be the precursor of the hormone isolated from alkaline extracts.

NH_2-Terminal Groups of Bovine Growth Hormone: It was first shown by Li and Ash (3) that the NH_2-terminal groups of bovine growth hormone isolated from alkaline extracts were phenylalanine and alanine and that they were present in an equimolar ratio. It was therefore surprising to find that growth hormone prepared by the three stages extraction method contained the theoretical quantity of NH_2-terminal alanine, whereas the phenylalanine was replaced to a variable extent by methionine. In the upper half of Table 5 are shown the NH_2-terminal groups of several such preparations of growth hormone and of growth hormone prepared by the method of Li (14). In addition to Ala, Phe, and Met, small but significant amounts of Glu and Ser were also invariably detected. Hydrazinolysis (19) of the hormone preparations listed in Table 5 showed that all contained 0.95 to 1.06 μM of COOH-terminal phenylalanine per μM of hormone. No other COOH-terminal groups were detected regardless of the

TABLE 5. RESULTS OF NH_2-TERMINAL ANALYSES OF GROWTH HORMONE

Growth hormone preparation no.	μM NH_2-terminal group per μM growth hormone*				
	Ala	Phe	Met	Glu	Ser
Li GH XIX-18-1	.91	1.08	0	0	0
XIV-44-c5	.92	.25	.94	.19	.16
XIV-39-2	.93	.21	1.00	.15	.10
XIV- 7-c6	1.07	.29	1.00	.30	.13
XIV-47-c7	.72	.59	.75	.17	.13
XIX-26- 4 {pH 4.0 H_2O 17 hrs. at 5° / pH 7.5 M/4 AS 17 hrs. at 5°	.85	.88	.24	.11	.15
XIV-16- 7 {1 hr. pH 5.5 H_2O / 2 hrs. pH 5.5 M/4 AS	.83	.32	.74	.20	.20
XIX-20-c7 {2 hrs. pH 5.5 H_2O / 2 hrs. pH 5.5 M/4 AS at 20°	1.02	.13	1.08	.29	.34
XIX-24-c6 {2 hrs. pH 5.5 H_2O / 22 hrs. pH 5.5 M/4 AS at 20°	.94	.15	1.00	.22	.33

* Calculated on basis of molecular weight of 45,000 and corrected for loss of DNP-amino acids during hydrolysis. Method of NH_2-terminal analysis employing dinitrofluorobenzene has been detailed in reference 26.

variation in NH_2-terminal composition. Moreover, all of the hormone preparations had a potency between 0.9 and 1.2 times the U.S.P. reference growth hormone and none were significantly different in potency from the reference at 95% confidence limits.

There was no correlation between the amounts of fast and slow components in the electrophoretic patterns discussed above and the content of NH_2-terminal methionine. This was proven by NH_2-terminal analysis of the growth hormone samples referred to in Table 4. Although the amount of slow component increased from 40 to 67% after 28 hours of incubation at pH 8.2, there was no detectable change in the content of NH_2-terminal Ala, Met, or Phe.

In attempting to account for the formation of "methionyl" growth hormone[6], it was assumed that the most favorable conditions for its formation existed in one of the multiple extraction stages. Accordingly, the extraction schedule was revised so as to omit extraction at pH 5.5. The ground pituitaries were extracted with M/10 $(NH_4)_2SO_4$ at pH 4.0 for 16 hours at 5°, thereby removing the major part of the TSH, LH and proteinase I. The residue was resuspended in M/4 $(NH_4)_2SO_4$ and extracted overnight at pH 7.5. The resulting extract was employed for the isolation of growth hormone by exactly the same procedures as already described. The hormone isolated in this manner contained only 0.24 μM of methionine suggesting, therefore, that the formation of Met-GH is indeed dependent upon extraction at pH 5.5 rather than pH 4.0 or 7.5. That the formation of Met-GH

[6] Henceforth abbreviated as Met-GH. The growth hormone containing only NH_2-terminal phenylalanine and alanine is referred to as Phe-GH.

occurs at pH 5.5 is further supported by the isolation of the hormone from glands which have been extracted only at pH 5.5 for the periods of time and temperature shown in Table 5. It is notable that the formation of Met-GH at pH 5.5 is quite rapid since only 1 hour of extraction with water and 2 hours with M/4 $(NH_4)_2SO_4$, plus a total centrifugation time of 3 hours still yielded a large amount of Met-GH (0.74 μM). This is remarkable in view of the fact that the entire extraction and isolation processes have been performed at 2 to 5 C. Isolation of the hormone from two stages of extraction at pH 5.5 in which the second stage was performed at 22° for either 2 or 22 hours yielded somewhat more NH_2-terminal methionine (1.08) and correspondingly less NH_2-terminal phenylalanine. Under these conditions only 0.13 to 0.15 μM of NH_2-terminal phenylalanine was present and represented the lowest quantity which could be obtained by modifying the conditions of extraction with M/4 $(NH_4)_2SO_4$ at pH 5.5. either with respect to time or temperature.

Ultracentrifugal analyses of growth hormone preparations containing various ratios of NH_2-terminal methionine and phenylalanine were performed at pH 2.39 in 0.1 M NaH_2PO_4–0.04 M H_3PO_4. All of the preparations shown in Table 5 yielded a single symmetrical boundary with an S of 3.5, which is in good agreement with that for phenylalanyl growth hormone of bovine origin described by Li and Pedersen (20). Analysis for methionine (21) in preparations containing 0.13 μM of NH_2-terminal phenylalanine and in growth hormone prepared by the method of Li (14) yielded a value of 2.1% methionine or 7 residues for a molecular weight of 45,000. That the molecular weight of the Met-GH is very close to that of the phenylalanyl hormone is also supported by the absence of more than one component or even significant asymmetry in the ultracentrifuge patterns of preparations shown to be mixtures of the two types of hormones by NH_2-terminal analysis. Finally, the fact that a mean of 0.95 moles of NH_2-terminal alanine per mole of the mixed hormone is obtained for a molecular weight of 45,000 supports the close similarity in molecular weight between the methionyl and phenylalanyl hormones.

Hydrolysis of Peptides Containing Methionine: In the preceding text it has been assumed implicitly that the methionyl-GH is derived in some manner from phenylalanyl-GH which is native to the pituitary and that the methionyl NH_2-terminal group is attributable to GH rather than to an impurity. The data of Table 5 which shows that the amount of Met-GH is inversely related to the amount of Phe-GH strongly supports these assumptions. In attempting to account for the formation of Met-GH from Phe-GH, either proteolysis or transpeptidation can be invoked. In the latter instance, a methionyl NH_2-termanal peptide in the crude extract could be transferred to the NH_2-terminal phenylalanine of GH by a transpeptidation mechanism similar to that demonstrated by Zioudraw *et al.* (22) for insulin, glycinephenylalanineamide and cathepsin C. However, in view of

the appreciable amount of proteolytic activity observed at pH 5.5 in crude pituitary extracts by Adams and Smith (2), it would appear that proteolysis might be a more likely mechanism which would account for the formation of Met-GH. To test this possibility, synthetic peptides containing methionine were tested for hydrolysis by the three extracts obtained from beef pituitaries. If methionine containing peptides (23) were found to be attacked at the amino side of methionine, then it would be reasonable to account for the formation of methionyl-GH by proteolysis.

The hydrolysis of CH_3CO-Ser·Tyr·Ser·Met·Glu (NH_2), Ser·Met·Glu (NH_2), Met·Glu (NH_2) and CBZ-Met·Glu (NH_2) at pH 5.5 by beef extract A is shown in Table 6. The hydrolysis was followed by the ninhydrin method of Moore and Stein (24) and the amino groups which were liber-

TABLE 6. HYDROLYSIS OF METHIONINE PEPTIDES BY BEEF EXTRACT A AT PH 5.5

Time of incubation, minutes	μM leucine equiv. formed per μM peptide		
	CH_3CO Ser·Tyr·Ser·Met·Glu (NH_2)	Ser·Met·Glu (NH_2)	Met·Glu (NH_2)
20	.23	.19	.002
40	.51	.35	.002
60	.63	.54	.005
120	.86	.79	.016
180	.89	.96	.020

Digest: 0.6 μM of the peptides and 0.5 mg. lyophilized beef extract A per ml. of 0.5 M $NaC_2H_3O_2$—$HC_2H_3O_2$ buffer at pH 5.5. Aliquots of 0.2 ml. were removed at the indicated time and analyzed by the Moore and Stein ninhydrin method (24), employing leucine as a standard.

ated are expressed in terms of leucine equivalents. The acetylated pentapeptide and the tripeptide yielded 0.89 and 0.96 new amino groups after three hours of incubation at 37°. Met·Glu (NH_2) was only slightly hydrolyzed, and CBZ-Met·Glu (NH_2) not at all. One dimensional paper chromatography was performed on separate digests obtained at pH 5.5 but in the absence of buffer salts. Employing the solvent system n-butanol-$HC_2H_3O_2$-H_2O (120:30:60) as recommended by Smith (25), the pentapeptide yielded a strong ninhydrin reactive spot corresponding to Met. Glu (NH_2) and trace spots corresponding to Met and Glu (NH_2). The tripeptide, Ser·Met·Glu (NH_2), yielded strong spots corresponding to Ser·Met and Glu (NH_2) and minor spots of Ser and Met·Glu (NH_2). The dipeptide, Met·Glu (NH_2) yielded traces of its component amino acids. Pure samples of Glu (NH_2), Met·Glu (NH_2) and Ser·Met·Glu (NH_2) were chromatographed simultaneously on the paper with the digests and served to identify the various ninhydrin spots. The distance of migration relative to Glu (NH_2) was 1.16 for Ser, 2.56 for Ser·Met·Glu (NH_2), 2.84 for Met·Glu (NH_2), 3.68 for Ser·Met and 3.81 for Met. Descending chromatography was employed on Whatman No. 1 paper and the lower edge was serrated to allow the solvent to drip off in order that Ser·Met and Met would be ade-

quately resolved. Identification of CH_3CO Ser·Tyr·Ser on the chromatograms was not attempted. Confirmation of the identity of the Met·Glu (NH_2) and Ser·Met spots was obtained by staining with iodoplatinic acid and periodate (25). The identity of the various reaction products in the digests were also confirmed by paper chromatography employing ethanol-H_2O-NH_3 (80:10:10). In neither solvent system were any unknown spots present which might suggest the occurrence of transpeptidation. The hydrolysis of the various peptides at pH 5.5 is summarized below where solid and broken arrows indicate major and minor reactions, respectively:

1. CH_3CO·Ser·Tyr·Ser·Met·Glu (NH_2) ⟶ CH_3CO·Ser·Tyr·Ser + Met·Glu (NH_2)
   ⇣
   Met + Glu (NH_2)

2. Ser·Met·Glu (NH_2) ⟶ Ser·Met + Glu (NH_2)
   Ser·Met·Glu (NH_2) - - - → Ser + Met·Glu (NH_2)

3. Met·Glu (NH_2) - - - → Met + Glu (NH_2)

The occurrence of reaction 1 indicates that beef extracts contain an enzyme capable of hydrolyzing a Ser·Met bond to yield a peptide with NH_2-terminal methionine, namely methionylglutamine. It is, therefore, reasonable to assume that the same enzyme is probably responsible for the formation of methionyl-GH from phenylalanyl-GH and that the derived hormone arises by hydrolysis and not transpeptidation.

The hydrolysis of Ser·Met·Glu (NH_2) by extract A at pH 5.5 can occur on either side on the methionine residue as evident from the presence of Ser·Met and Met·Glu (NH_2), although the latter is present in much smaller quantity than Ser·Met. This result suggests that two different enzymes may be involved in the hydrolysis of Ser·Met·Glu (NH_2). Data supporting this interpretation are summarized in Table 7. When the digestion of Ser·Met·Glu (NH_2) was performed at pH 4.0, cleavage occurred at the Ser·Mer- bond to yield Ser and Met·Glu (NH_2). The presence of Met and Glu (NH_2) in the digest is interpreted to mean that the Met·Glu

TABLE 7. HYDROLYSIS OF METHIONINE CONTAINING PEPTIDES AT DIFFERENT pH VALUES BY BEEF EXTRACT A*

Hydrolysis of Ser·Met·Glu (NH_2)		Hydrolysis products of peptides†		
pH	μM leucine equivalents per μM Ser·Met·Glu (NH_2)	Ser·Met·Glu (NH_2)	Met·Glu (NH_2)	CH_3CO·Ser·Tyr·Ser·Met·Glu (NH_2)
4.0	0.49	Ser+Met·Glu (NH_2) + Met+Glu (NH_2)	[Met+Glu (NH_2)]	Met·Glu (NH_2) +[Met+Glu (NH_2)]
4.5	0.64			
5.0	0.68			
5.5	0.48	Ser+Met·Glu (NH_2) + [Ser·Met+Glu (NH_2)] +[Met+Glu (NH_2)]	[Met+Glu (NH_2)]	Met·Glu (NH_2) +[Met+Glu (NH_2)]
6.0	0.71			
6.5	0.72			
7.0	0.61	Ser·Met+Glu (NH_2)	None	[Ser+Met·Glu (NH_2) +Ser·Met+Glu (NH_2)]

* Extracts B and C yielded results closely similar to A, but are omitted because of their very low level of activity.
† Enclosures within brackets indicate small or trace amounts of product.

NH_2) which is formed initially undergoes hydrolysis to its constituent amino acids. At pH 7.0, cleavage occurred at the -Met·Glu (NH_2) bond to yield only Ser·Met and Glu (NH_2). Table 7 also shows the amount of hydrolysis of Ser·Met·Glu (NH_2) in the range between pH 4.0 and 7.0. The minimum which occurs at pH 5.5 has been consistently observed in different preparations of extract A and further supports the hypothesis that two different enzymes are operative in the hydrolysis of Ser·Met·Glu (NH_2) with their optima for hydrolysis of this peptide at pH 4.5 to 5 and at pH 6 to 7, respectively. The former enzyme appears to be responsible for hydrolysis of the Ser·Met- bond and the latter for the -Met·Glu (NH_2) bond so that at pH 5.5 both peptides, Ser·Met and Met·Glu (NH_2), would form due to the overlap of activities of the two enzymes at this pH.

At pH 4.0 and 5.5 the hydrolysis of the acetylated pentapeptide proceeds readily to yield the dipeptide, Met·Glu (NH_2). At pH 7.0 however, only a small amount of hydrolysis occurred as indicated by trace quantities of the following products which were identified chromatographically: Ser, Ser·Met, Met·Glu (NH_2) and Glu (NH_2). Hence, the -Tyr·Ser- bond can also be hydrolyzed and may well be the rate limiting step for the formation of Ser·Met·Glu (NH_2) which is in turn hydrolyzed further to Ser·Met and Glu (NH_2) as shown for the tripeptide at pH 5.5 in Table 7.

The existence of proteolytic enzymes in pituitary extract A which are capable of hydrolyzing Ser·Met, Met·Glu (NH_2) and Tyr·Ser bonds may be therefore invoked to account not only for the formation of NH_2-terminal methionine in growth hormone but also the minor, but persistent amounts of NH_2-terminal Glu and Ser noted in Table 5. While the presence of such enzymes in the pituitary does not prove that the latter two amino acids are NH_2-terminal in Met-GH rather than being due to impurities, it nonetheless points to the latter possibility. That these groups are probably NH_2-terminal in Met-GH tends to be supported by their increase by elevated temperature or prolonged incubation during extraction at pH 5.5 and by the inability to reduce them in the isolated hormone by further purification. However, until the purified enzymes are available for testing on isolated GH, this point must remain undecided.

The formation of methionyl-GH and the hydrolysis of methionine peptides suggests that other proteolytic enzymes exist in the pituitary in addition to proteinases I and II originally described by Adams and Smith (2). The following preliminary observations support the existence of these separate enzymes. The pH optimum of the hydrolysis of Ser·Met·Glu (NH_2) is at pH 5 to 6 which does not correspond to the pH optima for the hydrolysis of denatured hemoglobin by proteinase I or II, whose optima are at pH 4.0 and 8.2 respectively. Highly purified pituitary proteinase I of sheep origin (26) is without effect on Ser·Met·Glu (NH_2) at pH 4.0 or 5.5 when tested at concentrations 2 to 100 times greater than that found in the corresponding beef extract A. The absence of hydrolysis by sheep

proteinase I cannot be ascribed to species specifity of the sheep enzyme since sheep extract A is also capable of hydrolyzing the tri- and pentapeptides in the same manner as described for beef extract A. When the three stage extraction is performed in the manner outlined, but the second extraction stage (pH 4.0, M/10 $(NH_4)_2SO_4$) is performed at 22° for 16 hours rather than at 2°, the digestion is so extensive at pH 4.0 that no trace of GH activity can be detected in any of the extracts or fractions. Moreover, under these conditions less than 10% of the usual yield of protein can be obtained from extract C, i.e., the third extract obtained with M/4 $(NH_4)_2SO_4$ at pH 5.5. With regard to possible identity of the postulated enzymes with proteinase II, it should be noted that considerably less Met-GH was obtained from extracts prepared at pH 7.5 as compared to pH 5.5 (Table 5), hence, the postulated enzymes are probably not identical with proteinase II since the optimum of the latter is at pH 8.2. Moreover, hydrolysis of Ser·Met·Glu (NH_2) and the acetylated pentapeptide is negligible at this or higher pH values.

Observations on Sheep Growth Hormone: The sheep GH obtained from sheep extract C differs from beef GH in several important respects. On electrophoresis at pH 4.0, the sheep preparation showed 90% of a component with a mobility of 5.5×10^{-5} cm.^{2} sec^{-1} v^{-1} and 10% of another component with a mobility of 3.4×10^{-5} cm.^{2} sec^{-1} v^{-1}. On prolonged storage in the lyophilized state or in alkaline solutions as described for beef GH (Table 4), no conversion to the slower moving electrophoretic component could be observed as in the case of beef GH. NH_2-terminal analysis of the sheep GH yielded 0.9 μM each of Phe and Ala, and COOH-terminal analysis 0.9 μM of Phe per μM of the hormone which is in close agreement with the data of Papkoff and Li (27). Moreover, variations in the time and temperature of extraction at pH 5.5 changed neither the amount of NH_2-terminal amino acids nor the electrophoretic pattern. These results suggested that the proteinase responsible for the formation of Met-GH in beef glands might be absent in sheep pituitaries or else the sheep growth hormone molecule may not be susceptible to their action. When sheep extract A was tested for hydrolytic action on Ser·Met·Glu (NH_2) and on the acetylated pentapeptide at the pH values used with beef extract shown in Table 7, essentially the same results were obtained. Hence, the lack of formation of Met-GH in sheep extracts is not due to the absence of the appropriate proteinases but must be due to the absence of susceptible peptide bonds or to some difference in configuration of the molecule which renders the bond unaccessible to the enzyme.

DISCUSSION

The distribution of FSH, LH, TSH and GH in the three successive extracts presents a relatively constant pattern for beef, sheep and pig pituitaries. Thus, extract A is essentially an FSH concentrate when prepared

from sheep or pig glands; extract B is primarily a TSH and LH concentrate, extract C is a growth hormone concentrate and extract D is a prolactin concentrate. An exception to this pattern is observed with pig extracts A and B. Here the major proportion (59 percent) of the LH appears in extract A, although the highest specific activity of LH is found in extract B as is true for beef and sheep pituitaries. Proteinase I is mainly present in extract A, with the exception that in pig extracts the proteinase activity is equally distributed between extracts A and C. Proteinase II appears primarily in extract C from all three species of glands, although pig material again presents an exception to the pattern in that a considerable proportion is also present in extract A. It should be emphasized that this pattern of extraction of the hormones and proteinases has been obtained with fresh pituitaries which had been stored at $-20°$ anywhere from a month to several years. It is not known what effect repeated thawing and freezing, prior dehydration and defatting with acetone, or lyophilization of the whole glands, may have on the extraction pattern.

A consideration of the results obtained by the serial extraction of pituitary glands shows that the solubilization of the different hormones and proteinases into the three types of extracts is not as complete as would be ideally desirable. However, from the practical standpoint, the following advantages are evident. Serial extraction results in a fairly selective concentration of certain hormones and proteinases in the various extracts in contrast to the usual alkaline extracts (1, 8–12) in which nearly all of the hormones and other soluble proteins are obtained together. Hence, fairly complete separations of certain hormones or groups of hormones are already achieved in the extracts themselves, which thereby permits an appreciable simplification in the subsequent stages of purification of the hormones and proteinases. Finally, the method of serial extraction permits a more complete and economical utilization of pituitary glands since it is possible to obtain starting materials from a single lot of glands which can be used for the further purification of five hormones and at least two proteinases; FSH, LH, TSH, GH, prolactin and proteinases I and II.

Met-GH obtained from beef pituitaries appears to be an artifact of the three stage extraction procedure which is performed at pH values where proteolytic enzymes, previously undescribed, are optimally active. Alkaline extraction such as employed by Li (14) precludes an opportunity for action by these proteinases and the Phe-GH obtained from such extracts must be the precursor of Met-GH. The partial degradation of the Phe-GH to Met-GH is not accompanied by any loss in potency as determined from assays employing hypophysectomized rats. The Met-GH has also been tested in monkeys for possible activity in primates, but like the phenylalanyl hormone, the methionyl derivative was incapable of stimulating the release of unesterified fatty acids into the blood.

An interesting question concerning the methionyl-GH is whether only

one or several methionyl bonds of the 7 present in bovine (28) GH are cleaved by the enzyme. Since ultracentrifugal analysis yielded a single symmetrical component with a sedimentation constant equal to that of Phe-GH it is very likely that only one methionyl bond was split. A random splitting of methionyl bonds would probably result in ultracentrifugal heterogeneity. In addition, the results of analysis for methionine revealed that Met-GH still contains seven residues of the amino acid per molecular weight of 45,000. Hence, it can be concluded that only a single methionine bond is hydrolyzed and that it is located nearer to the NH_2-terminal than the other methionine residues.

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SUBTOTAL HYSTERECTOMY AND OVARIAN FUNCTION IN GILTS[1,2]

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ABSTRACT

This investigation deals with ovarian function in gilts following subtotal hysterectomy during the luteal phase of the cycle. In 7 groups of 5 gilts per group the following parts of the uteri remained in the animals: Group 1, posterior half of cervix; Group 2, body and cervix; Group 3, anterior one-fourth of right horn and posterior half of cervix; Group 4, anterior half of right horn and posterior half of cervix; Group 5, posterior half of right horn including body and cervix; Group 6, anterior halves of right and left horns and posterior half of cervix; and Group 7, sham operations. Nine of 10 gilts in Groups 1 and 2 did not show estrus during a 120-day period following the estrus prior to experimental treatment. Three of 5 gilts in Group 3 did not return to heat during a 70 day period following the estrus prior to surgery. All gilts in Groups 4, 5 and 6 had average estrous cycle intervals of 25 days as compared to 21 days for the same animals prior to hysterectomy. The sham-operated gilts in Group 7 had average estrous cycle intervals of 21 days. These findings suggest that retrogressive transformation of active corpora lutea into corpora albicantia during non-fertile cycles is dependent upon a stimulus from the uterine horns.

Persisting corpora lutea (marked) and no heats were observed in gilts in which corrosives and irritants were injected into the lumen of the right uterine horn and body during the luteal phase of the cycle. The left horn was removed.

FSH activity was significantly lower ($P < 0.05$) 2 days after the onset of estrus than at any other stage of the cycle or pregnancy investigated. Fifty days after removal of the uterus FSH activity was significantly lower ($P < 0.05$) than 25 or 110 days after surgery. Differences in LH activity were not statistically significant at any of the stages investigated. Lactogenic hormone potency increased during the cycle and advancing stages of pregnancy. Following removal of the uterus, the quantity of this hormone was greater than during pregnancy.

There was a marked decrease in the progesterone concentration in the corpus luteum 110 days after subtotal hysterectomy. *In vitro* synthesis of this

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hormone by luteal tissue was observed 25, 50 and 110 days after removal of the uterus.

GILTS are useful experimental animals for investigations dealing with mammalian reproduction. They are litter bearing and polyestrous throughout the year. The average length of estrous cycles is 21 days and of pregnancy is 113 days. Ovulation is spontaneous and corpora lutea are functional during the non-fertile cycle. Furthermore, various organs of the reproductive tract provide adequate amounts of tissue for morphological, physiological and biochemical studies.

This investigation deals with the effect of subtotal hysterectomy on ovarian function in the gilt. Duncan *et al.* (1) have reported that subtotal hysterectomy prolonged the functional life span of gilt corpora lutea as indicated by progesterone content and the capacity of luteal tissue to synthesize this hormone *in vitro*.

EXPERIMENTAL PROCEDURE

Thirty-five crossbred gilts ranging from 6 to 8 months of age were used as experimental animals. Estrous behavior was checked daily with vasectomized boars. The occurrence of normal estrous cycles was observed in all animals before placing them on experiment. Subtotal hysterectomy in this study consisted of the surgical removal of the uterus from the tubo-uterine junction of each horn to the middle of the cervix. In some experimental groups, relatively smaller portions of the uterus were removed. Oviducts and the posterior half of the cervix remained intact in all animals in this study. The latter was not removed because of its relatively inaccessible pelvic location in the gilts.

In 6 groups of gilts with 5 animals per group the following parts of the uteri were excised: Group 1, right and left horns including body and anterior half of the cervix; Group 2, right and left horns; Group 3, the left horn, posterior three-fourths of right horn including body and anterior half of cervix; Group 4, the left horn, posterior half of right horn including body and anterior half of cervix; Group 5, the left horn and anterior half of the right horn; and Group 6, the posterior halves of the right and left horns including body and anterior half of cervix. In sham-operated gilts, Group 7, laparotomies were performed and the uteri were manipulated but remained intact. Figure 1 shows the reproductive tract of the gilt and the sketches indicate portions of the uterus retained by animals in various groups following subtotal hysterectomy.

Each animal was administered intramuscularly 10 mg. of tranquilizer (Trilafon, Schering Corp.). This was followed by injection of 60 cc. of 2% Procaine (Abbott Laboratories) in the area of the incision. A ventral mid-line laparotomy was used. All hysterectomies and sham operations were performed during the luteal phase of the cycle, days 8 through 11, and the corpora lutea were marked with carbon for subsequent identification at slaughter. Sterile animal charcoal was placed in each corpus luteum with a sterilized dissecting needle. Length of the uterine horns was measured with a piece of sterile cotton cord at the time of surgery. Furthermore, number, size and characteristics of the corpora lutea were recorded. A "Polyotic Oblet" (American Cyanamid Co.), containing 500 mg. of tetracycline hydrochloride, was placed in the peritoneal cavity prior to suturing.

Gilts were checked daily for estrus following surgery. Two heat periods were observed post-surgery and the gilts were slaughtered from days 8 through 11 following the second estrus in Groups 2, 4, 5, 6 and 7. Animals in Groups 1 and 2 not showing estrous

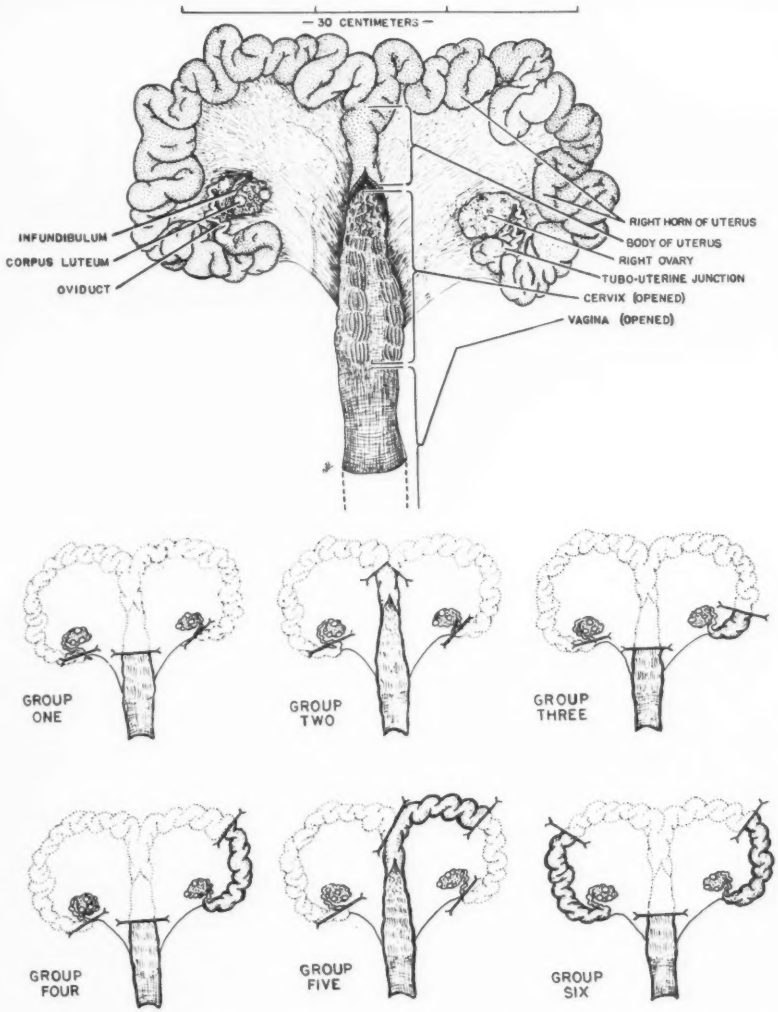

FIG. 1. Reproductive tract of gilt. The shaded areas in the smaller sketches indicate portions of the uterus retained by individuals in different experimental groups.

behavior after surgery were slaughtered 119 to 121 days following the estrus prior to experimental treatment. Gilts in Group 3 were slaughtered 68 to 78 days following the estrus either prior to or after subtotal hysterectomy. Pituitary glands from animals in Group 1 were removed and frozen immediately. Each anterior lobe was individually evaluated for FSH, LH and lactogenic hormone activities by bioassay methods previously described by Day *et al.* (2).

The initial progesterone concentration in luteal tissue and the capacity of the tissue to synthesize this hormone *in vitro* were determined 25, 50 and 110 days after removal of

the uterus (from mid-cervix to tubo-uterine junctions) in 11 gilts. Procedures for quantitative estimation of progesterone and for evaluating *in vitro* progesterone synthesis have been described by Duncan *et al.* (3).

RESULTS

The influence of various types of subtotal hysterectomies and sham operations on the occurrence of estrus are summarized in Table 1. Table 2 shows the effect of uterine extirpation on the weight of the corpus luteum. FSH, LH and lactogenic hormone activities of pituitaries of gilts at different stages of the estrous cycle, pregnancy and following subtotal hyster-

TABLE 1. EFFECT OF SUBTOTAL HYSTERECTOMY ON ESTRUS

Group	Part of uterus remaining in animal	Number of gilts	Estrous cycle interval following operation	
			First	Second
			days	
1	Posterior half of cervix	5	none	
2	Body and cervix	4	none	
		1	26	31
3	Anterior one-fourth of right horn and posterior half of cervix	3	none	
		2	28 ± 1[a]	none
4	Anterior half of right horn and posterior half of cervix	5	32 ± 6	23 ± 0
5	Posterior half of right horn, body and cervix	5	25 ± 2	25 ± 2
6	Anterior halves of right and left horns and posterior half of cervix	5	23 ± 1	24 ± 2
7	Sham operation, uterus intact	5	21 ± 0	22 ± 0

[a] Mean $\pm$ standard error.

ectomy are presented in Table 3. Table 4 is a summary of the effect of removal of the uterus on luteal progesterone.

DISCUSSION

Gilts with only the posterior half of the cervix intact did not show estrus during a 120-day period following the last heat prior to surgery (Group 1, Table 1). Furthermore, estrus was not observed for the same period of time in 4 of 5 animals with the body and cervix present (Group 2). One animal in this group, however, did show a first estrous cycle interval of 26 days following subtotal hysterectomy and a second of 31 days. This gilt was slaughtered 11 days after the second estrus. The quantity and gross morphology of the residual reproductive tract in this animal was comparable to that present in the 4 which did not return to estrus before slaughter at 120 days. The carbon-marked corpora lutea were present in all 9 animals in Groups 1 and 2 which did not show estrous behavior.

TABLE 2. EFFECT OF SUBTOTAL HYSTERECTOMY ON LUTEAL WEIGHT

Group[a]	Average slaughter weight	Average number of corpora lutea per animal	Average corpus luteum weight
	lbs.		mg.
1	385	13	455 ± 25[b]
2	387	13	400 ± 28
3	385	11	453 ± 28
4	325	15	635 ± 68
5	310	13	522 ± 32
6	322	16	693 ± 76
7	301	13	622 ± 88

[a] Five animals in each group; see Table 1 for group descriptions.
[b] Mean ± standard error.

Du Mesnil Du Buisson and Dauzier (5) reported persistence of corpora lutea for 117 days with ovulation occurring about 200 days after the last estrus in hysterectomized sows which were pregnant and non-pregnant at the time of surgery. Spies *et al.* (6) hysterectomized 4 gilts in early luteal phase of the cycle and observed no heats prior to slaughter 32, 71, 72 and 119 days after last estrus.

The results obtained from gilts in Groups 1 and 2 suggest that the presence of either the posterior half of the cervix or the body and cervix,

TABLE 3. FSH, LH AND LACTOGENIC HORMONE ACTIVITIES OF ANTERIOR LOBE OF PITUITARY GLANDS FROM GILTS AT DIFFERENT STAGES OF CYCLE, PREGNANCY AND POST-HYSTERECTOMY

Stage of gilts[a]	Number of gilts	Average slaughter weight	Dry weight anterior lobe of pituitary	FSH assay: uterine weight[b]	LH assay: ventral prostate weight[c]	Lactogenic hormone per mg. anterior pituitary
days		lbs.	mg.	mg.	mg.	I.U.
Estrous cycle						
2	6	217	38	85 (6)[d]	43 (3)[d]	0.2 (6)[d]
10–12	6	220	39	125 (6)	61 (4)	0.3 (6)
17–19	6	249	38	131 (6)	56 (3)	0.4 (6)
Pregnancy						
25	6	286	41	116 (6)	64 (3)	0.4 (6)
85	6	299	50	131 (6)	67 (6)	0.5 (6)
Post-hysterectomy						
25	3	270	51	115 (3)	62 (3)	1.3 (3)
50	2	290	57	67 (2)	56 (2)	0.8 (2)
110	5	385	68	101 (5)	62 (5)	1.2 (5)
Control rats						
1) Cycle and pregnancy				38 (8)[e]	44 (5)[e]	
2) Post-hysterectomy, 25 and 50 days				35 (8)	44 (5)	
3) Post-hysterectomy, 110 days				32 (6)	44 (6)	

[a] Cycle and pregnancy data from Duncan (4).
[b] Uterine weights represent the mean of 2 levels with total doses of 4.6 and 9.3 mg.
[c] Ventral prostate weights represent the mean of 2 levels with total doses of 3.5 and 10.5 mg.
[d] Number of pituitaries assayed.
[e] Number of rats.

in the majority of animals, does not appear to influence the regression of corpora lutea under conditions of the experiments reported. However, these data show that the absence of non-gravid uterine horns has a similar influence on the life span of the corpora lutea as the presence of pregnant horns. In 1923, Loeb (7) reported complete or almost complete extirpation of the uterus of the guinea pig prolonged luteal function.

The relationship of different portions of the uterine horns and the occurrence of estrous cycles following subtotal hysterectomy is indicated by results obtained in Groups 3, 4, 5 and 6. Three of 5 gilts, with the anterior one-fourth of the right horn and the posterior half of the cervix intact, did not show estrus following surgery (Group 3). These 3 animals were slaughtered 72, 76 and 77 days after the heat prior to experimental treatment and carbon marked corpora lutea were present in each gilt. First estrous cycle

TABLE 4. EFFECT OF SUBTOTAL HYSTERECTOMY ON PROGESTERONE CONCENTRATION IN CORPORA LUTEA

Number of days to slaughter after removal of uterus	Number of gilts	Average number of corpora lutea	Average weight of total luteal tissue per animal	Progesterone concentration		
				Initial	After 2 hrs. incubation	Increase
			gm.		μg./gm.	
25	4	9	4.3	80	125	45
50	2	16	5.8	64	110	46
110	3	10	4.9	38	104	66
110	2	16	6.9	22	—	—

intervals of 26 and 29 days were observed following subtotal hysterectomy in 2 gilts in Group 3. However, a second estrus was not detected in these 2 gilts and they were slaughtered 78 and 68 days after the first post-surgery heat. The average estrous cycle interval in Groups 4, 5 and 6 was 25 days (range 19 to 57) as compared to 21 days (range 20 to 23) for the same animals prior to experimental treatment. Carbon marks in the stroma of the ovaries as well as unmarked corpora lutea were observed at slaughter in 2 gilts in Group 3 and in all animals in Groups 4, 5 and 6. The 5 sham-operated gilts in Group 7 maintained normal estrous behavior and estrous cycle intervals following surgery. A delay in estrous cycle intervals of 3 to 4 days in Groups 4, 5 and 6 as compared to Group 7 would suggest that a decidual reaction may occur in the gilt when the endometrium is cut at the time of subtotal hysterectomy. Data summarized in Table 1 suggest that retrogressive transformation of active corpora lutea to corpora albicantia during each non-fertile cycle is dependent upon a stimulus from the uterine horns. The results from the majority of animals (3 of 5) in Group 3 showed that the anterior quarter portion of one uterine horn was insufficient uterine tissue to induce regression in corpora lutea. However, this quantity of tissue may be considered

threshold in the two animals in Group 3 which did show estrous cycles. Larger amounts of either one or both horns were adequate for luteal regression as shown by Groups 4, 5 and 6. A specific or localized anatomical region does not seem to be involved in the pig. Gross mammary development was not observed in any of the gilts in these 7 experimental groups.

In the case of the guinea pig, the life span of the corpora lutea was somewhat prolonged when one-third or one-fourth of the uterus was allowed to remain intact (7). Morphologic changes in the endometrium which occur during the different phases of the estrous cycle are probably related to changes in physiologic activity of the endometrium. The absence of this endometrial stimulus in gilts in Groups 1, 2 and 3 could explain the persistence of functional corpora lutea and the absence of estrous cycles.

Loeb (8) has discussed the similarity between the effect of pregnancy and of hysterectomy on the guinea pig corpus luteum. He suggested the possibility that in pregnancy a functional inactivation of the uterine mucosa is responsible, at least partially, for the observed effects on the corpus luteum. According to Bradbury *et al.* (9), in some species it appears that there may be luteolytic substances, especially in the endometrium, which hasten the involution of the corpus luteum in non-fertile cycles. Results from a study of factors influencing *in vitro* synthesis of progesterone by Duncan *et al.* (1) indicate that endometrial filtrates from days 12 and 13 of the estrous cycle increased *in vitro* synthesis by gilt luteal tissue whereas filtrates from days 16 and 18 showed an inhibitory effect on hormone production. However, these factors may influence luteal tissue activity *in vitro* but may not be involved with corpus luteum function *in vivo*.

The results obtained from the investigation presented here suggest that a stimulus may be produced by the uterus during the non-fertile cycle which could be directly or indirectly responsible for the regression of the corpus luteum. This stimulus could be a hormone or a local neurohumoral substance. A hormone produced by the uterus and transported by the blood and lymph may have either a direct effect on the ovaries or an indirect action by way of the pituitary. A neurohumoral substance produced by the endometrium may influence nervous activity and thereby modify ovarian function through pituitary control. Moore and Nalbandov (10) have demonstrated neurogenic effects of uterine distention on the estrous cycle of the ewe. Hansel and Wagner (11) have observed that uterine dilatation modifies the length of the bovine cycle. Metabolic utilization of luteal progestins by the uterus may also influence the life span of the corpus luteum. The anterior one-fourth of one uterine horn (Group 3) may approach the quantity of uterus necessary to metabolize the ovarian progestins and thereby reduce the circulating level of these substances. This would be followed by luteal regression and subsequent gonadotrophin release. Du Mesnil Du Buisson (13) partially hysterectomized gilts during

the luteal phase of the cycle. When the length of the remaining anterior part of one uterine horn exceeded 26 cm. (about one-fourth of a horn) estrous cycles continued, however, when less then 26 cm. of one horn remained intact estrus was inhibited. In the latter case a unilateral regression of corpora lutea was observed on the ovary on the side of the conserved uterine fragment whereas corpora lutea retained their typical functional appearance on the opposite ovary. However, a consistent unilateral luteal regression was not observed on the ovary on the side of a part of a remaining uterine horn in groups 3, 4 and 5 in this study reported here.

In a preliminary study it was observed that the life span of corpora lutea was extended in animals following the injection of an irritant or corrosive in the lumen of the uterine horn. The left horn was removed from the tubo-uterine junction to the body of the uterus in 4 gilts on day 11 or 12 of the cycle. The lumen of the right horn and body was injected with one of the following: (1) 125 cc. of 10% tannic acid; (2) 50 cc. of 12.5% phenol; (3) 70 cc. of Bouin's fluid; or (4) 130 cc. of 10% silver nitrate. These animals were slaughtered 47 or 48 days after the estrus prior to treatment. Histologic examination of uterine tissues indicated that both tannic acid and Bouin's fluid caused necrosis of the endometrium. Following phenol the glandular epithelium was cuboidal whereas the epithelium of the endometrial stroma was a low columnar type. Silver nitrate induced chronic ulceration (endometritis) and atrophy of glandular epithelium. It is of interest that persistent corpora lutea are present in cows with pyometra. Estrous behavior was not observed in these gilts during this period and the carbon-marked corpora lutea were present at the termination of the experiment. These findings indicate irritants and corrosives modify uterine function in such a way as to bring about a persistence of corpora lutea and as a result no estrous cycles occur. In contrast to these observations, estrus was observed in animals with only the posterior half of the right uterine horn, body and cervix intact (Table 1, Group 5). This horn of these animals was not treated with chemicals. Hansel and Wagner (11) have reported alteration of bovine estrous cycles and luteal function following infusion of seminal and preputial fluids as well as seminal sediment. These findings suggest a modification of uterine function.

There was no significant difference at the 5% level between Groups 1, 2, 3, 4, 5 and 7 (Table 2) with respect to average corpus luteum weight. Corpora lutea in Group 6 were significantly heavier than in Group 2 ($P < 0.05$). Nine of 15 gilts in Groups 4, 5 and 7 had an average of 16 corpora lutea of which 7 in each gilt were hemorrhagic. The increase in organ weight of Groups 4, 5, 6 and 7 was due to this hemorrhagic condition.

The bioassay methods employed did not allow for a direct quantitative measurement of FSH and LH activities of the individual pituitary glands. However, the uterine weight response was considered to give an approxi-

nate FSH potency and the ventral prostate weight was used to estimate LH activity (Table 3). The FSH activity of the anterior pituitary 2 days after onset of estrus was significantly lower ($P < 0.05$) than any other stage of the estrous cycle or pregnancy investigated. At 25 days and 110 days post-hysterectomy FSH activity was comparable to that observed during the estrous cycle and pregnancy. However, FSH activity was significantly lower at 50 days than at 25 or 110 days post-hysterectomy.

There was no detectable LH activity 2 days following estrus and differences among reproductive stage means were not statistically significant in this assay ($P < 0.05$). The removal of the uterus did not appear to influence LH activity of the anterior pituitary. Results presented in Table 3 show an increase in lactogenic hormone potency with advancing stages of the estrous cycle and pregnancy. The quantity of this hormone following hysterectomy was greater than during the cycle or pregnancy. Duncan *et al.* (1) have previously reported that ovine lactogenic hormone did not have luteotrophic activity on swine corpora lutea as indicated by *in vivo* and *in vitro* experiments.

A decrease was observed in the initial progesterone concentration in luteal tissue as the time interval increased following removal of the uterus (Table 4). This may indicate either a reduction in steroid production or an increased rate of synthesis and release of progesterone from corpora lutea. The initial progesterone concentration in gilt luteal tissue 25 and 50 days following hysterectomy was comparable to that previously observed during the luteal phase of the cycle and early pregnancy (3). Rowlands and Short (12) reported increased luteal size and progesterone concentration following hysterectomy in guinea pigs. However, the concentration in gilts 110 days following removal of the uterus was comparable to that found at 96 and 102 days of gestation. Luteal tissue from animals after hysterectomy retained its capacity to synthesize progesterone *in vitro* as indicated by data in Table 4. The greatest increase was observed in tissue 110 days following the removal of the uterus. The increases at 25, 50 and 110 days after hysterectomy were comparable to those reported for luteal tissues from pregnant animals (3).

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THYROID-PITUITARY FEEDBACK: DIRECT OR INDIRECT? A COMPARISON OF THE EFFECTS OF INTRAHYPOTHALAMIC AND INTRAPITUITARY THYROID AUTOTRANSPLANTS ON PITUITARY THYROIDECTOMY REACTIONS IN THE RAT[1]

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ABSTRACT

Thyroid autotransplants to the hypophyses of thyroidectomized rats reversed anterior pituitary thyroidectomy reactions in a graded fashion. Near the grafts the reaction was strongly inhibited, whereas remote pituitary regions were so unaffected they could serve as intrinsic controls. Intermediate regions exhibited intermediate thyroid hormone effects. Implants of control tissue had no such specific effects. Thyroid, but not control, implants in the hypothalamus produced a diffuse, less striking inhibition of the thyroidectomy reaction. The interpretation is offered that this inhibition resulted from a direct thyroid effect on the pituitary thyrotrophs and acidophiles. Evidence is presented favoring this supposition over the alternative idea that the hypothalamus forms part of a slowly reacting thyroid-pituitary feed-back circuit.

THE concept of thyroid-pituitary feed-back as a homeostatic mechanism is well known. It rests upon the assumption that there are specific receptor cells which react to their environmental thyroid hormone (TH) concentration in such a way that thyrotrophin (TSH) secretion is increased by TH deficiency and lowered by TH excess. Neither the nature nor location of these hypothetical TH receptors has yet been reported.

Interest in the idea that such receptors might be located in the hypothalamus was provoked by observations that specific thyroid (1) and pituitary (2, 3) reactions to TH deficiency can be impaired by hypothalamic lesions. Euler and Holmgren (4) first examined this possibility by comparing the effects of minute intrapituitary and intrahypothalamic thyroxine injections on TH release in the rabbit. They found that only the intrapituitary injections inhibited TSH release. Other experiments have shown that systemic thyroxine administration can depress or abolish TSH secretion despite the disruption of hypothalamic-pituitary mech-

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anisms by hypophyseal transplantation (5), pituitary stalk section (6), or large hypothalamic lesions (7, 8).

These collected findings do localize a thyroid-pituitary feed-back cutoff mechanism at the pituitary level, but do not reveal its intrapituitary locus. Moreover, they do not eliminate the possibility that TH may also act *via* the hypothalamus, for two reasons. Firstly, even repeated negative observations cannot disprove the existence of a thyroid-hypothalamic-pituitary feed-back mechanism. Secondly, the cited experiments were all done on animals which initially were secreting TSH at either normal or subnormal rates. However, the hypothalamic TSH influencing mechanism seems to be involved most obviously, if not exclusively, in supernormal TSH secretion (7, 9). Even if it could be assumed that TH acts solely by an adenohypophyseal route in suppressing relatively low rates of TSH secretion, it does not necessarily follow that it uses the same route in inhibiting the higher rates which are elicited by thyroidectomy or goitrogen treatment.

In contrast to the amassed negative evidence are the studies of Yamada (10–12), which demonstrate that intrahypothalamic thyroxine microinjections can, indeed, inhibit TSH secretion in the rat. Curiously, although Yamada found intrahypothalamic and intrapituitary thyroxine administration to be about equally effective, the intrapituitary injections acted far more rapidly ($<\frac{1}{2}$ *vs.* 2–16 hours) in arresting initially normal thyroid activity (10, 12). He found further that thyroxine, injected intrahypothalamically, prevented goitrogenesis (11) but failed to report whether intrapituitary injections also could prevent this pituitary reaction to prolonged systemic lack of TH.

We have attempted to compare the effects of locally elevated intrahypothalamic and intrapituitary TH concentration on the microscopic pituitary thyroidectomy reactions of the rat. In this species, under chronic conditions, pituitary structure varies markedly at different rates of TSH secretion (13, 14). The histological approach has allowed us to observe, for the first time, a direct action of TH at the pituitary cellular level. It has also yielded other observations which bear pertinently on Yamada's findings and argue against his interpretation (12) that dual (hypothalamic and hypophyseal) TH receptor systems are involved in thyroid-pituitary feed-back.

PROCEDURES

All experiments were done on adult female Holtzman rats, which were thyroidectomized under ether anesthesia. To effect local TH replacement in the brain or hypophysis, one or more small fragments of the ablated glands were implanted intracranially a few (5–20) minutes after thyroidectomy. These autotransplants were delivered stereotaxically, as described by Flerkó and Szentágothai (15) in their study of comparable ovarian grafts.

Single thyroid fragments were put in (or near) one adenohypophyseal lobe (usually the left) whereas transplants to various parts of the hypothalamus consisted of an even

number of pieces (2–8), placed bilaterally in approximately symmetrical positions near the midline.

Controls were of 2 types. Some (unimplanted) were merely thyroidectomized, or thyroidectomized and deprived of a small piece of control tissue (ovary, liver, muscle or salivary gland). Others (implanted) also received an intracranial autotransplant of control tissue.

Three consecutive series (H, I, J) of about 60 rats each were operated upon. They were then supplied freely with commercial diets[3] and tap water. The H series received the least dietary iodine, J the most. Only 89 rats survived the experimental period. Of these, 12 were discarded on the basis of radiometric evidence of either grossly incomplete thyroidectomy or poorly localized grafts.

On the 27th postoperative day, each survivor was injected intraperitoneally with about 25 μc of carrier free NaI^{131}. It was killed with ether 21 to 27 hours later. The following were collected for radiometric and/or microscopic study: a) thyroidectomy site (pharynx, larynx, trachea, esophagus, infrahyoid muscles and fascia, removed *en bloc* between the hyoid bone and manubrium); b) hypophysis (in most cases, along with its surrounding dura mater and adjacent parts of the Vth nerves, sphenoid, occipital and temporal bones and varying amounts of adherent pharyngeal roof tissue); brain, which was trimmed, after partial fixation, to rectangular blocks containing c) anterior hypothalamus and d) posterior hypothalamus (as well as the overlying regions).

Block (a) was placed at once in a test tube, with 1 ml. of water, for subsequent radioactivity measurement. The other specimens were fixed for about 6 hours before being trimmed and transferred to counting tubes with 1 ml. of fixative. Hypophyses were fixed in SUSA-picric acid (9:1), brains in 10% neutral formalin. Block (a) was counted in every case, but the activities of the other blocks were measured only if they were suspected of containing thyroid transplants or were needed to establish "tissue background" (the magnitude of radioactivity in the absence of such grafts).

The specimens, together with appropriately diluted 1 ml. aliquots of the I^{131} solution administered, were counted by deep-well scintilometry 7–14 hours after autopsy. The concurrent "dose counts" obviated decay correction. After background correction, specimen and dose counts were used to calculate I^{131} "uptake," or more precisely, Relative Activity (RA) with a subscript (a, b, c or d) denoting the specimen. RA = specimen activity $\times 2 \times 10^4 \div$ dose activity. This value usually approximated the actual count /minute because calculated dose activity was usually about 2×10^7 counts/minute.

After being counted, thyroidectomy site blocks were discarded. The other tissues were returned to jars containing adequate (about 50 ml.) volumes of fixative, in which they remained for 3 days. The prolonged SUSA treatment served to decalcify juxtahypophyseal bone. Further processing and embedding (in 56° C Tissuemat) were done routinely. Two sets of frontal, 10 μ, interrupted serial sections (every 7th and 8th, 9th and 10th) were prepared from each implant-bearing brain block. One set was stained with thionine, the other with hematoxylin-eosin. Complete, 4 to 10 μ serial sections through the hypophyses were cut in the horizontal plane and stained with Gomori's trichrome and aldehyde-fuchsin (AF).

OBSERVATIONS

Thyroidectomy Controls

Neck count activity (RAa) varied over a wide range, suggesting that some residual thyroid tissue was often present. In 37 control rats, these

[3] Series H was fed Big Red dog blocks, stated to contain 0.2% of iodized salt. Series J got Purina Fox Chow (0.5% iodized salt). Series I was transferred from Big Red to Purina after about 2 weeks.

residua rarely appeared to impair thyroidectomy cell development, although more sensitive indices of TH effect such as the maintenance or restoration of pituitary acidophilic (α) and/or aldehyde fuchsinophilic (β) granulation generally reflected their presence (Table 1). RAa did not always correlate closely with the intensity of such granulation. There were cases, especially in series H, in which the adenohypophyses appeared devoid of granules even though neck counts were high. The radiometric method thus appears extremely sensitive, particularly in animals on a low iodine diet. On the other hand, some controls exhibited a considerable degree of pituitary α and β granulation, but low neck activity. This discrepancy, perhaps attributable to ectopic thyroid rests, suggests that such rests could be detected reliably only by more extensive radiometric exploration. In thyroidectomized guinea pigs, Kochakian and Cockrell (16) have found functional thyroid tissue not only in such logical remote sites as the tongue and mediastinum, but even in salivary gland!

From the foregoing, it is evident that the degree of thyroidectomy incompleteness could not always be ascertained. Nevertheless, it seems reasonable to assume that the incidence of seriously incomplete thyroidectomies would be about the same in control and experimental rats ($<10\%$). This assumption is necessary to interpret the findings in rats with hypothalamic implants, but it is not needed in considering the engrafted hypophyses. The (predominantly unilateral) pituitary grafts exhibited sharply limited, concentric zones of effect, so that peripherál pituitary

TABLE 1. RESIDUAL NECK RADIOACTIVITY (RAa) AND HISTOLOGIC CRITERIA OF THYROIDECTOMY INCOMPLETENESS IN 37* CONTROL RATS

RAa	Pituitary indices of TH titer†											
	H series‡				I series				J series			
	IV	III	II	I	IV	III	II	I	IV	III	II	I
2.5– 5						X0	X00	X		XX0	XXX	
5.1– 10		0				X00	0	X0		0		
10.1– 25				X0		XX						
25.1– 99						0		X		X		
100–200	X	0		X00	X0	X						
>200		X	X									

* 21 implanted (X) and 16 unimplanted (0) controls. See text (Observations-Control Implants to Brain) for further description. Each symbol (X or 0) = 1 rat.

† I—No evidence of residual TH. Thyroidectomy cells well developed (Figs. 5, 6B) and seen throughout anterior lobe (i.e. in every high power field). Acidophiles and AF+secretory granules absent.

II—Slight TH effect. Thyroidectomy cells as in I. Acidophiles small and sparse. AF+secretory granules absent or very rare.

III—Moderate TH effect. Thyroidectomy cells similar to those in I and II. Acidophiles numerous and often large. AF+cells present sparsely or in moderate numbers.

IV—Pronounced TH effect, seen in 3 controls. In 2, thyroidectomy cells were somewhat reduced in size and absent from the periphery of the gland, a picture comparable to Fig. 6A. In 1 rat, thyroidectomy cells were not even seen and the gland resembled that of a euthyroid rat. Acidophiles were conspicuous in all 3.

‡ The 3 series (H, I, J) have been tabulated separately to illustrate the influence of dietary iodide levels (lowest in H, highest in J) on RAa.

regions—particularly the contralateral anterior lobes—could serve as intrinsic controls.

Thyroid-engrafted hypophyses

Fig. 1A indicates various graft positions in relation to the hypophysis. The numbers of I^{131}-concentrating thyroid grafts found in each (approximate) position were: a-2, b-5, c-5, d-3, e-4, f-2. The grafts in the f position, and one in the a position, failed to alter the microscopic pituitary reaction to thyroidectomy. The other 18 had distinctly localized effects; the thyroidectomy reaction appeared unmodified in regions remote from the grafts. Each engrafted hypophysis thus served as its own control.

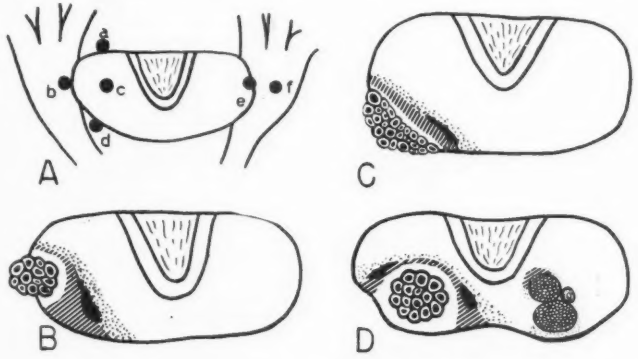

FIG. 1. A—Scheme of rat hypophysis and trigeminal ganglia in horizontal section. The various graft positions are indicated by black dots labelled a–f. In B, C and D, thyroid grafts are shown at left. In D, an ovarian graft is shown on the right. The clear zones (I) adjacent to the thyroid grafts were populated only by small chromophobes and shrunken acidophiles. More peripherally, the striped zones (II) indicate regions containing normal or large acidophiles, but no thyrotrophic forms. The black zones (III) mark the regions where hypertrophic AF+cells, as well as acidophiles, were concentrated. Stippled zones (IV) are transitional to the more peripheral (clear) "control" regions.

Within the affected regions, adenohypophyseal structure varied according to distance from the graft (Figs. 1 B, C, D and 2B). The pituitary tissue adjacent to each effective transplant resembled that seen in rats treated with very large amounts of thyroxine (14, 17). It formed a zone (I) exhibiting only small chromophobes and shrunken acidophiles (Figs. 2C, 3F, 4C-bottom). This zone gave way to a region, farther removed from the graft, in which large, brilliantly staining acidophiles abounded (arrows, Figs. 2D, 3E, 4C—middle section). The acidophiles formed a dense band, prominent under low magnification, at the circumference of zone I. The inner part of this band (zone II) appeared to be devoid of thyrotrophs; in its outer parts (zone III) were seen the "thyroidectomy cells" closest to the graft. These cells were generally smaller, less vacuolated and less

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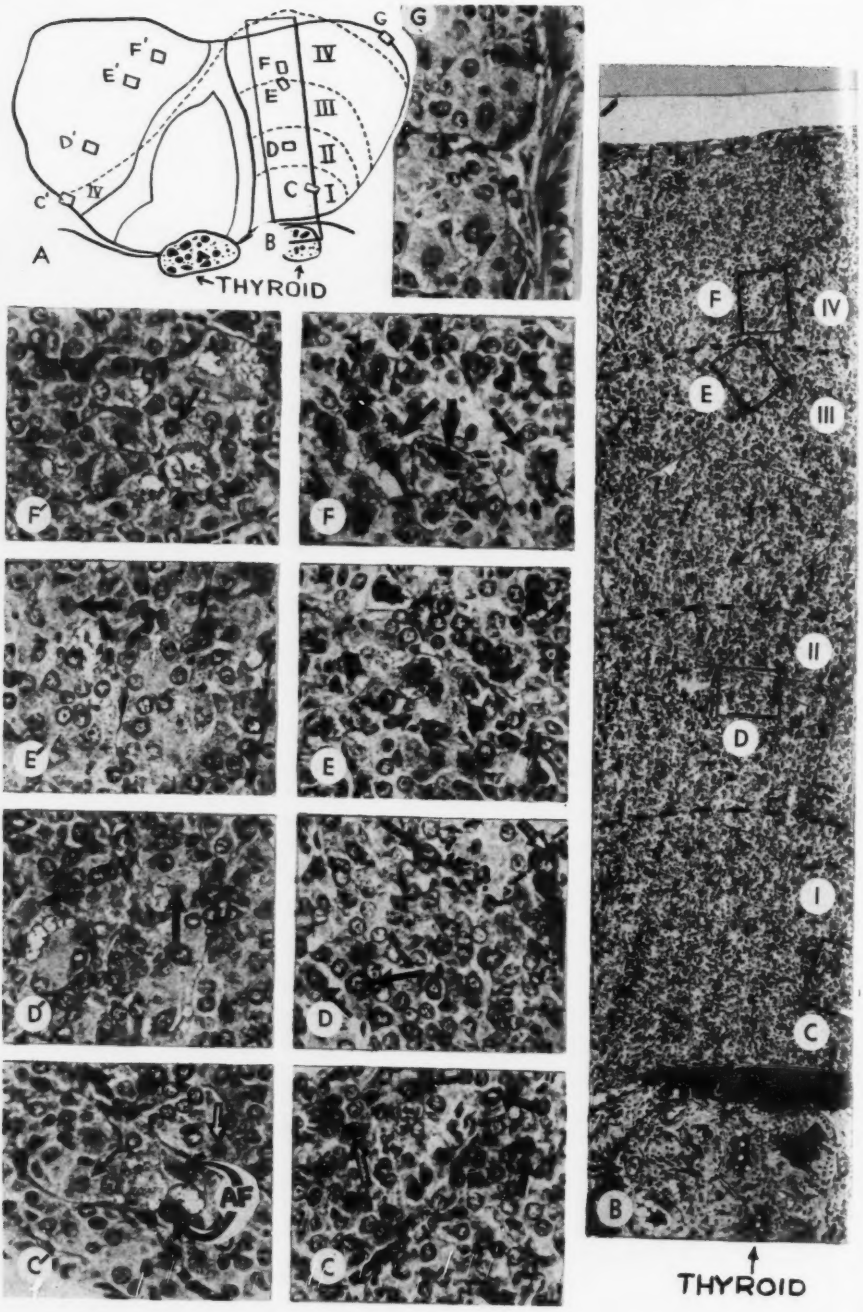
A
THYROID
G
F
E
D
C
B
AF
IV
III
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THYROID

numerous than the thyroidectomy cells in more remote regions (Figs. 2E *vs.* E′, 4C-upper left *vs.* E). Frequently (in 15 of the 18 affected zones) a number of them contained AF stainable material (Fig. 2E and F).

These AF+ cells did not conform in shape to the classical β cell (18, 19) or thyrotroph (20–22). Instead, they resembled the hypertrophic, vacuolated, AF+ cells which often are pathognomonic of mild TH deficiency (13). Such cells were numerous and ubiquitous in the adenohypophyses of 2 of the 3 inadequately thyroidectomized controls. They were not always numerous in the thyroid-engrafted hypophyses but, when present, they distinctly were concentrated in the outer part of the acidophilic band. Their presence made the transition between the inner and outer parts of this band quite sharp.

The transition between the acidophilic band and the "control" regions of the anterior lobe was gradual. The concentration of acidophiles and hypertrophic β cells decreased as typical thyroidectomy cells increased in size and numbers. At a variable distance from the graft, an approximate limit could be assigned to the effect (Figs. 2A, 3B, 4B). Beyond this limit very few AF-positive cells or acidophiles were discernible and the thyroidectomy reaction appeared classical (23) or only mildly impaired.

The effectiveness of a graft seemed to be correlated more with its position than with its I^{131} uptake which is not, at best, a direct index of TH secretion. In these experiments (in which dietary iodine content varied) it could serve reliably only as a qualitative indicator of functional thyroid tissue.

The affected zones were not spherical. They extended least far anteriorly and reached farthest posteriorly (perhaps also laterally). Their medial

FIG. 2. Double thyroid graft in position a (accidentally placed adjacent to hypophysis). A—17× sketch showing graft placement, concentric zones I–IV (see legend Fig. 1) and loci of medium (86×) and high (380×) power views. B—Rectangle indicated in A, showing condition of graft and precise loci of Figs. C–F in zones I–IV. C—Zone I. Arrows point to shrunken acidophiles. C′—Comparable contralateral area with few acidophiles and many thyroidectomy cells. Some thyroidectomy cells in this region (zone IV?) contained AF+ secretory material (double arrow labelled AF). The majority contained only a dense spot of AF+ material in the Golgi region (single arrows). D—Zone II, inner part of acidophilic band. Arrows indicate large acidophiles. Clear area marked off in upper right corner is suggestive of early thyroidectomy forms. D′—Comparable contralateral area showing large vacuolated thyroidectomy cells with AF+ material visible only in the Golgi region (arrows). E—Zone III, outer part of acidophilic band—showing large vacuolated cells staining intensely with AF (black). F′—Transitional zone (IV) with larger, less intensely AF+ thyroidectomy cells (arrows), but fewer acidophiles. E′, F′—Comparable contralateral regions, entirely similar to D′. G—Region at extreme left posterior tip of adenohypophysis. This was the only area in the left lobe within which normal thyroidectomy forms could be found.

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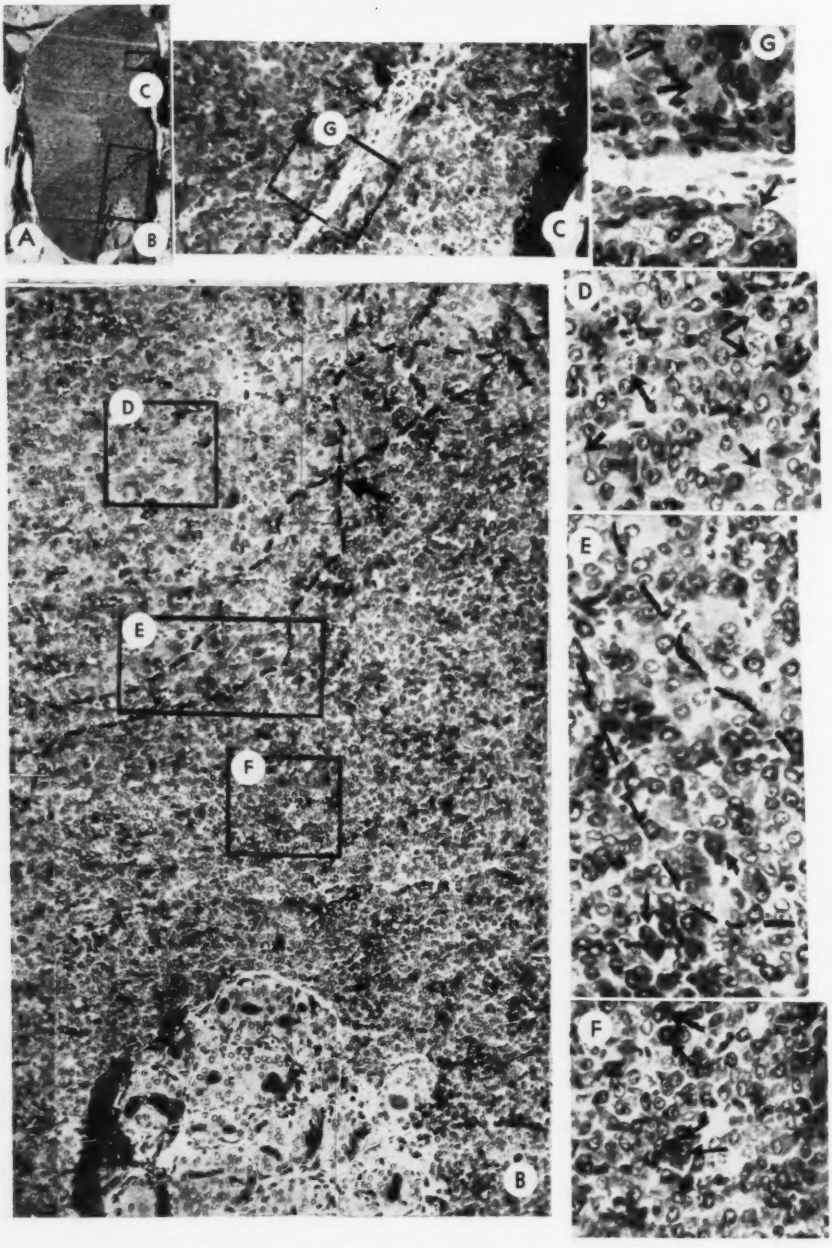


FIG. 3. Hypophysis bearing thyroid graft (position e) in left lobe and scar at site of resorption of liver implant in right lobe. A—10×—Indicates loci of B and C. B—138×—Shows affected and "control" zones, condition of graft and loci of Figs. D–F (330×). Dashed lines surround transitional region which is occasionally very narrow (arrows). Arrows in D indicate thyroidectomy cell vacuoles. Arrows in E and F indicate acidophiles. C and G—138× and 330×—Thyroidectomy cells (arrows) adjacent to implant scar.

borders usually appeared (in horizontal section) to have the characteristic streamlined shape depicted in Figs. 1, 3, and 4. These observations suggest that the anterior part of the hypophysis would be the most effective graft site, and this appears to be true (Fig. 2A). Even very large grafts in the posterior part of the gland (Fig. 1C) had surprisingly slight effects. It

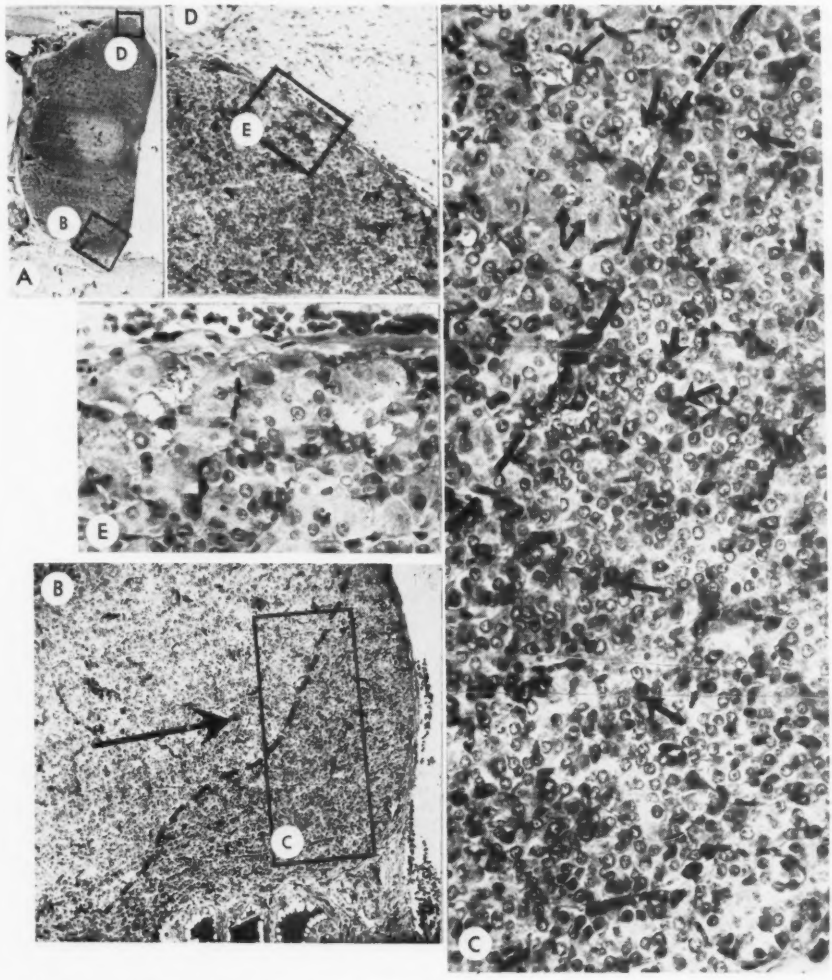
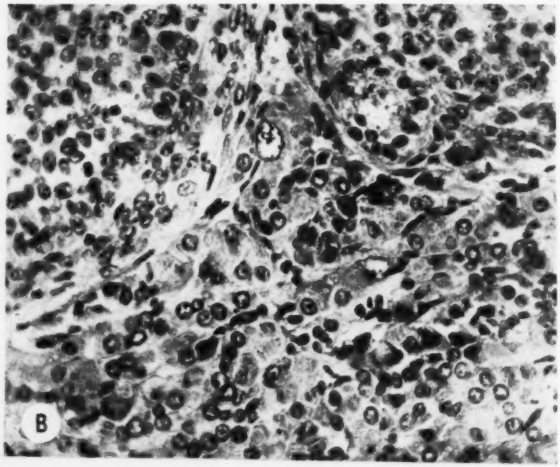


FIG. 4. Position b graft. A—10×—Shows loci of B and D. B—78×—Shows small thyroid graft, shape of affected zone and location of C. Large arrow indicates (presumed) direction of blood flow. C—330×—Arrows in affected zone indicate acidophile; in "control" zone they indicate thyroidectomy cell vacuoles. D and E—78× and 330×—Comparable contralateral region. Note numerous thyroidectomy cells at very edge of gland.

B
A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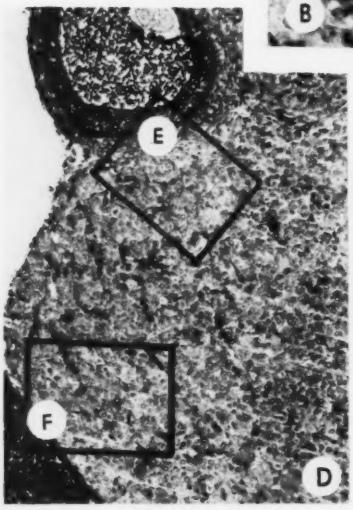
E
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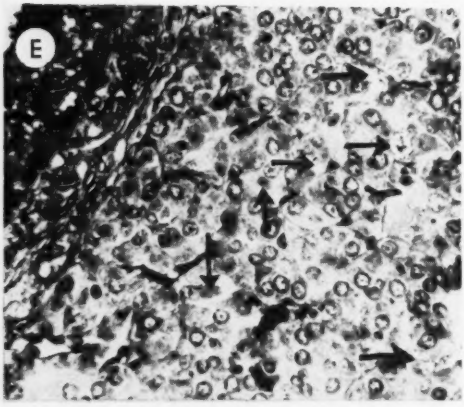
D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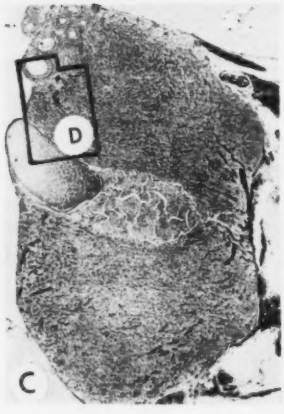
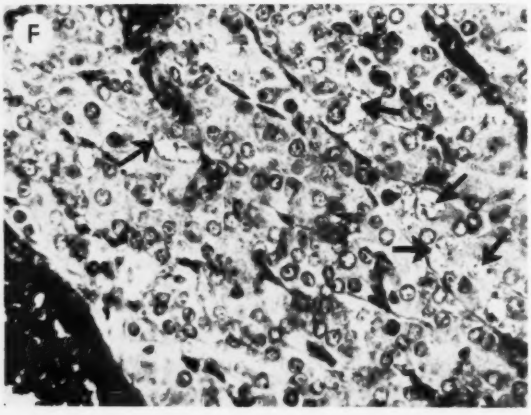

D
C


F

seems most probable that TH diffusion from the graft was greatly influenced by the antero-posterior flow of stalk vessel blood (Fig. 4). The failure of one of the two position a grafts to have any effect might have been due to inadequate contact between the graft and the stalk vessels, but this could not be assessed in these preparations.

Control tissue-engrafted hypophyses

Some implants of thyroid gland and ovary, and all of muscle, liver or salivary gland, were resorbed. A number left scars marking their positions, at the very edges of which thyroidectomy cells were abundant, whereas acidophiles were absent. Such scars were seen in glands which also contained effective thyroid grafts (Fig. 3).

Four ovarian grafts survived. One was in the right lobe of an adenohypophysis which bore an effective thyroid graft in its left lobe (Fig. 1D). This graft and one other contained corpora lutea, or luteinizing follicles; all four had follicles. Purely follicular grafts (Fig. 5) had no apparent influence on pituitary structure.

On the other hand, corpora lutea did modify the thyroidectomy reaction somewhat (Fig. 1D). Near luteal bodies (but not follicles in the same graft) large, well-granulated acidophiles were evident, but they were not nearly as numerous as those found in the zones affected by thyroid grafts. Thyroidectomy cells were not found quite so close to corpora lutea as to follicles, but the cells next to the corpora lutea were definitely larger and healthier looking than those adjacent to thyroid grafts. Moreover, AF+ cells were not seen near the ovarian grafts.

Since active-appearing follicular grafts had no effect, the separate effects of thyroid and luteal grafts on pituitary structure would appear to be specific. Further work would be needed to interpret fully the significance of the luteal effects.

Control implants to brain

Eight rats received fragments of ovary in the hypothalamus; 2 others were implanted with muscle. The implants did not survive, and only scars and necrotic masses could be found in serial sections.

FIG. 5. Ovarian grafts in hypophysis (purely follicular). A—18×—Two follicles in b position; rectangle shows locus of B. B—390×—Interfollicular tongue of pituitary tissue with large, fully vacuolated thyroidectomy cell in this intimate relation to ovarian tissue. C—18×—Mass of follicles at right anterior tip, large follicular cyst in neural and intermediate lobes. D—91×—Zone between implants, showing positions of E and F. E—Horizontal arrows point to vacuoles, vertical arrows to AF+ Golgi zones, in thyroidectomy cells. F—Many vacuolated (arrows) thyroidectomy cells. Virtually no acidophiles.

Despite the fact that 5 of these 10 had RAa values greater than 100, only one of the 5 showed even slight impairment of thyroidectomy cell development itself. These 10 rats (and 11 with intrapituitary implants of control tissue only) have therefore been pooled with unimplanted controls in Table 1.

Thyroid grafts to brain

Active (RAc or d from 24 to 306) thyroid grafts were found in the anterior hypothalamus in 11 rats, and in the posterior hypothalamus in 8. Six of the former and 4 of the latter exhibited some reduction in thyroidectomy cell size and/or concentration. Several others showed borderline effects, so that only 2 anterior and 2 posterior implants could clearly be seen to be ineffective. Neck counts were not very high in these 2 groups since several of their number had been previously discarded on the basis of high RAa values. Except for 2 rats with RAa values of 118 and 42 (with no pituitary evidence of TH effect!) the highest RAa was 25.

The incidence of pituitary manifestations of TH effect in these rats greatly exceeded that for the controls (in which higher neck radioactivity was tolerated). Thus, although any individual pituitary TH effect might be attributable to extrahypothalamic thyroid tissue, there can be little doubt that the findings for the group as a whole indicate that both anterior

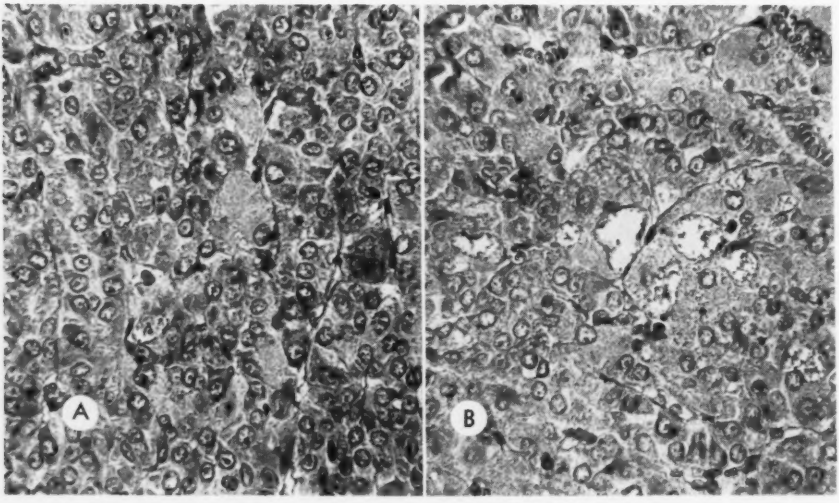


FIG. 6. A—Adenohypophysis of rat bearing thyroid implant in anterior hypothalamus (see Fig. 7). Numerous acidophiles (dark cells) and small thyroidectomy cells characterize the picture. AF+ thyroidectomy cells were also seen, but are not illustrated in this photograph. RAa = 3.7; RAb = 1.7; RAc = 104; RAd = 0.3. B—Control gland of same series. Note virtual absence of acidophiles and much larger size and greater abundance of thyroidectomy cells. (475×).

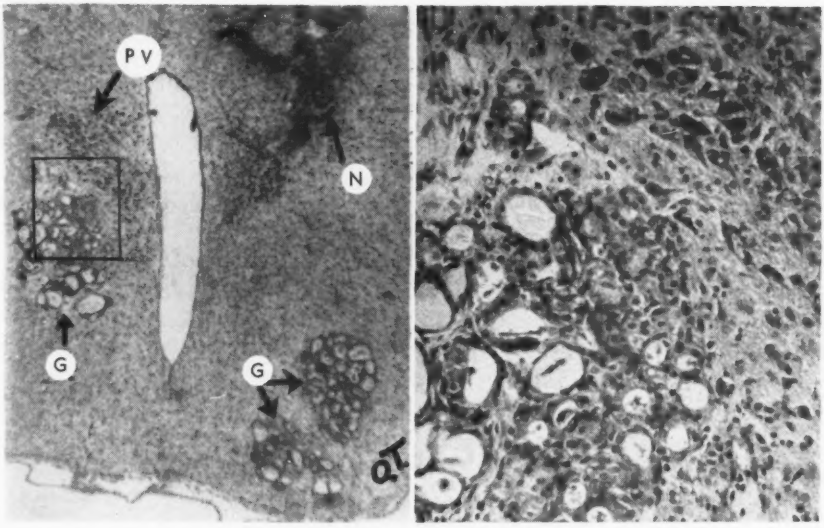


FIG. 7. Sections showing location of thyroid graft in rat whose hypophysis is depicted in Fig. 6A. A—Low power view showing graft (G), paraventricular nucleus (PV), optic tract (OT), a necrotic mass extending up into thalamus, due either to degenerating implants or hemorrhage occasioned during implant placement (N) and region shown in B. B—View showing paraventricular nucleus (upper right) and condition of adjacent thyroid graft. Although some follicles appear moribund, several can be seen with healthy looking cells (RAc = 104). A left, B right.

and posterior hypothalamic grafts had some inhibitory effect on the thyroidectomy reaction.

The inhibition, not localized, was less marked than that which accompanied intrapituitary grafts. It was thus not always obvious. A typical example is contrasted with a control in Figure 7. The position and structure of the presumably responsible graft are shown in Figure 7.

DISCUSSION

These findings do not allow a quantitative comparison of the effectiveness of hypothalamic- and pituitary-implanted thyroid tissue. It may be that the less severe but widespread inhibition of the thyroidectomy reaction, in rats with thyroid-engrafted brains, actually represents an equal or even greater overall curtailment of TSH secretion than the striking but sharply localized inhibition seen adjacent to thyroid grafts in the hypophysis.

However, these experiments clearly show that TH inhibits the pituitary thyroidectomy reaction, both partially and completely, by directly affecting the thyrotrophic cells. They also suggest that this direct effect may be hindered considerably because of rapid removal of the TH by the pituitary

circulation, which sweeps it caudally so that it influences primarily the regions *behind* the graft.

This rapid flushing of TH from the hypophysis may, perhaps, explain Yamada's (12) observations: 1) The effective intrapituitary dose of thyroxine is greater than would be expected from dilution of the effective systemic dose; 2) Radioactive thyroxine, injected intrahypothalamically, largely disappears from the brain before any pituitary effect is evident (average delay—8 hours), but fails to accumulate in the hypophysis.

It seems reasonable that most of a sudden intrapituitary injection would be removed before it could act. On the other hand, smaller amounts of hormone, infused continuously *via* the portal or nutritive circulation and similarly swept away, would also be continuously replaced. From this it follows that intrahypothalamic TH, from a graft or an injection, might well reach the hypophysis as a long-lasting, low concentration infusion after first diffusing (at least partially) to the portal capillary bed of the median eminence. The diffusion delay is quite in keeping with Yamada's observation (10) that the effect of intrahypothalamic thyroxine on TSH secretion is delayed.

Since a direct inhibitory mechanism undoubtedly exists for both high and low initial TSH secretory rates, and since both Yamada's and our observations do appear consonant with the idea that intrahypothalamic TH administration could exert its effect *via* this direct mechanism, Yamada's suggestion (12)—that the delayed effect of intrahypothalamic TH injections reveals the existence of a slowly reacting neural TH feed-back system —appears unnecessary.

Acknowledgments

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A MANOMETRIC ASSAY OF INSULIN AND SOME RESULTS OF THE APPLICATION OF THE METHOD TO SERA AND ISLET-CONTAINING TISSUES[1]

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ABSTRACT

The use of the manometric measurement of the net gas exchange of rat adipose tissue to quantitate small amounts of insulin is described. The method is simple, does not require that insulin standards be run concomitantly and is capable of measuring quantities of insulin of the order of 0.01 μg. In addition, the method permits the response of the tissue to be observed throughout the time course of the procedure. The importance of this in the measurement of the insulin-like activity of certain biological materials is illustrated. Examples of the application of the technique to the measurement of the insulin-like activity of toad fish islet tissue, human insulinomas, and human sera are given. The interpretation of the results in the case of sera is complicated by the unusual behavior in activity that it exhibits upon dilution and upon storage in the frozen state.

IN EARLIER publications (1) (2) a simple and rapid manometric procedure for following the action of insulin *in vitro* upon adipose tissue was described. It is based upon the measurement of the net gas exchange of the tissue in a bicarbonate buffered medium containing glucose. The addition of insulin to such a system results in a marked liberation of CO_2 accompanied by a stimulation of glucose uptake (3) (4) and the formation of fat (3). The rate of CO_2 release as measured manometrically in terms of net gas exchange has been found to be a function of the insulin concentration within the range 10^{-5} to 10^{-3} units per ml. Thus the use of the procedure, as suggested earlier (1) for the assay of small quantities of insulin is an obvious one. Since then, considerable experience has been gained in the use of the procedure so that reproducible results can be obtained. In addition, the technique has been applied to sera and certain islet containing tissues in an attempt to quantitate their insulin-like activity. Though the interpretation of some of the results obtained, especially those with sera, is open to discussion, the method appears to offer certain advantages which warrant the presentation of the data at this time.

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PROCEDURE

It should be mentioned at the outset that attention to certain small details and precautions which are described is of importance if reliable and consistent results are to be obtained.

The epididymal fat pad of rats purchased from the Holtzman Company has been used exclusively for these studies though white adipose tissue from other sites (2) or other animals conceivably could be employed. The proper care and handling of the animals is of extreme importance if consistent results are to be obtained from day to day. Our animals have been housed in quarters in which the temperature (72–75°) and humidity have been automatically controlled. To insure a uniform feeding pattern the lighting is also automatic and is on continuously from 6 A.M. to 9:30 P.M. Animals have free access to water and Purina laboratory chow at all times. Care is taken not to excite, scare, or injure the animals prior to sacrifice otherwise erratic results may be obtained. It is advisable not to employ rats from a new shipment until several days have elapsed in their new quarters. Abnormal tissue behavior has also been noted when the rats have been brought a half hour or so before sacrifice to a laboratory where the temperature was 5–7° lower than the animal quarters. The animals are sacrificed by decapitation by the use of a miniature guillotine manufactured by the Harvard Apparatus Company. Rats sacrificed by a blow on the head or any other method which results in trauma has been less satisfactory in our hands. Rats in the weight range 150–225 grams (6–8 weeks old) are employed. Younger rats yield insufficient tissue while tissue from older rats is much less active (5).

Removal of Tissue

The need for care in the handling of tissue has been previously emphasized (1) (3). Our routine procedure is as follows: the animal's testes are forced gently into the abdominal cavity and a wide abdominal incision made with scissors, care being taken not to nick the bladder or intestines. A testis is grasped with forceps and lifted sufficiently to permit a hemostat to be placed on the fat pad at the point of juncture with the testis. With the fat pad held by means of the hemostat it is cut free from the testis, the epididymus and blood vessels, and lifted out of the abdominal cavity. Portions of tissue are then cut off the distal end with sharp scissors onto a small tared square of pliofilm and weighed on a torsion balance. It should be noted that the absolute weight of the tissue is not required in this method. Hence this step may be eliminated provided that the amount of tissue used per 1.5 ml. of medium falls within the 100–200 mg. range. We prefer to use only one portion weighing 100–150 mg. from each fat pad though two pieces from each pad may be easily obtained from rats weighing around 200 gm. The portion of the fat pad held by the hemostat is always discarded. The tissue is gently rolled from the pliofilm with the aid of a glass spatula onto a spoon shaped piece of cellophane made by slicing in half longitudinally a cellophane test tube (100×14 mm.) and trimming appropriately. The end of the spoon containing the tissue is then inserted into the neck of a Warburg vessel well past the ground glass surface and the tissue nudged into the medium in the flask with the aid of a glass spatula. Adipose tissue has a great tendency to stick to various surfaces including glass and since the action of insulin on adipose tissue appears to be on the cell membrane (*cf* 6) precautions against injury of the cell surface are advisable. The time between the removal of the tissue from the animal and placing it in the incubation medium (at room temperature) is kept to a minimum (1–2 minutes). No advantage appears to accrue by maintaining the temperature of the medium at 38° during the loading of the flasks.

Incubation medium

The incubation medium is the bicarbonate—salt solution of Krebs and Henseleit (7) but with one half the calcium recommended by these workers. This lower calcium con-

centration is employed since it more nearly approaches the value for ionized calcium in blood serum and also leads to the formation of less turbidity in the medium on standing. We have found that erratic results have been obtained when slightly turbid full strength calcium medium has been employed. The medium is gassed with 5% CO_2-95% air daily before use. This also helps to prevent precipitation of calcium salts and insures the proper pH for the tissue before the flasks are gassed. Glucose is added to the salt solution to yield a concentration of 360 mg. per 100 ml. Gelatin is added, except as noted, in the amount of 200 mg. per 100 ml. to prevent loss of insulin by adsorption on glass surfaces (*cf* 1). This amount of gelatin does not all dissolve at room temperature and the excess is allowed to settle out, or may be filtered off. The glucose and gelatin containing medium is made fresh daily. No attempt should be made to store this medium in the cold overnight since a turbidity may develop which will lead to low results.

Manometric procedure

The Warburg manometric apparatus is employed. We have routinely used vessels with a total capacity of 8–10 ml, having one sidearm with a vented stopper and no center well. The total fluid volume used in this sized vessel is 1.50 ml. When possible the preferred technique is to place 0.05 to 0.10 ml. of the solution to be assayed in the sidearm of the flask and 1.40–1.45 ml. of the glucose-gelatin medium in the main compartment. Larger vessels (with or without center wells) and correspondingly larger total fluid volumes may be employed if desired. After the tissue has been added to a flask it is placed on its manometer and inserted in a water bath maintained at 37.4° C. When all manometers and flasks are ready they are gassed simultaneously with 5% CO_2-95% air for about five minutes with slow shaking. The gas is bubbled through water before admission to the flasks. After an additional 10 minutes of shaking, readings are begun and are made at 10 minute intervals for a 30 to 40-minute period. During this period the net gas exchange should be zero or negative. A definite increasing positive pressure response is an indication that the tissue is behaving abnormally and that it is not suitable for assay purposes. The contents of the sidearm are then tipped into the flask and the pressure response followed at 10-minute intervals until a new rate is established and maintained for at least a 30-minute period. With small quantities of insulin the rate may continue to change with time for some 30–60 minutes after tipping. In most cases once a rate is established it is usually linear with time, provided that an adequate shaking rate is maintained, e.g. 120 per minute. However in dealing with serum or certain of its fractions the pattern of response may be abnormal as discussed below.

When the response to the solution to be assayed has been ascertained the flask is removed from the manometer and 0.05 ml. of a solution containing sufficient insulin (0.15 units for 1.5 ml. final fluid volume) to insure a maximum response by the tissue is added to the main compartment. The flask is replaced on the manometer, reinserted in the water bath and gassed as before. Readings are commenced ten minutes after gassing is completed and continued at 10-minute intervals until a linear rate is observed, usually 30 minutes. The assay is now completed. We have found it possible for one person to run concurrently a half dozen assays in 3–4 hours.

Calculation of results and some applications of the method

The calculation of the insulin content of the sample is best described by the use of examples. In Figure 1 is shown the assay of a toad fish islet tissue and of a human insulinoma. The net gas pressure change is plotted against time. In Experiment A in which toad fish islet was used the initial rate of pressure change was −3 mm. per hour. At the arrow marked 1, 0.05 ml. of an homogenate of toad fish islet tissue was added and a new rate of +53

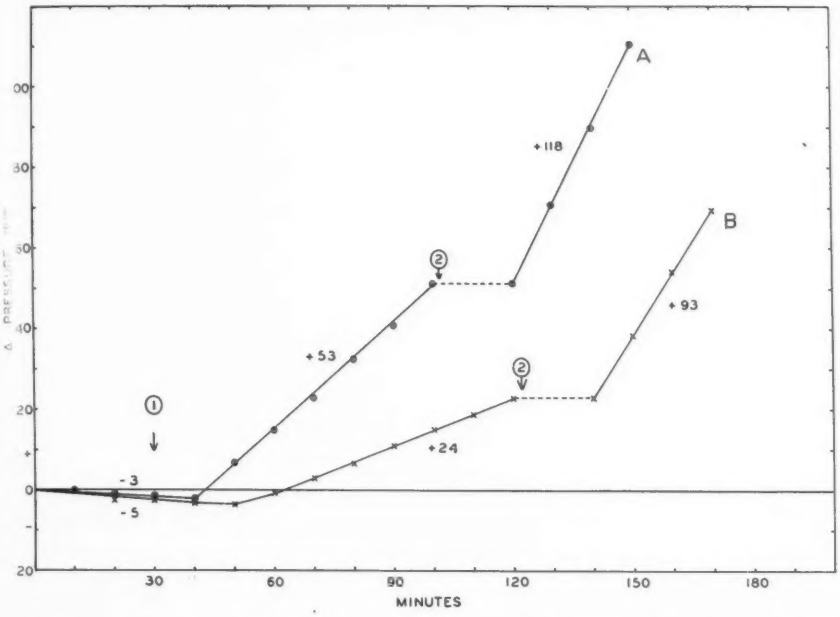

FIG. 1. *The assay of toad fish islet* (*A*) *and human insulinoma tissue* (*B*). Tissue was homogenized in the Krebs bicarbonate medium containing gelatin and diluted appropriately with the same medium. The precautions described in the text in connection with the data presented in Figure 2 were employed in making these dilutions and in measuring the samples for assay. The toad fish islet tissue assay was one of many performed in connection with an unpublished study carried out in collaboration with Drs. A. Lazarow and A. Renold. The human insulinoma tissue assay was one of a series performed for Drs. J. R. Brooks and D. F. Hickok. Tissue homogenate, 0.05 ml., was tipped in from the sidearm at arrow 1 and 0.05 ml. of a crystalline insulin solution (0.15 μ units) added at arrow 2. Numbers on graph refer to rates in terms of mm gas pressure change per hour per flask. The adipose tissue employed in each assay was obtained from different rats and hence the maximum rates are not to be inter-compared.

mm. per hour was established, the net change in rate being +56. At the arrow marked 2, the flask was disconnected and 0.05 ml. of a solution of crystalline insulin containing 0.15 units was added. The rate subsequent to this addition was +118 mm. per hour or a net change from the initial of +121. The response of the tissue to the toad fish islet extract is thus 56/121 or 46% of that obtained with a maximum amount of insulin. This value of 46% is now used to obtain the insulin content by means of the data presented in Figure 2.

The results shown in Figure 2 were obtained with a sample of crystalline insulin (Lot No. 466368) kindly furnished by Eli Lilly and Company. In each of the 49 experiments shown here the procedure was the same as that described in the text and illustrated in Figure 1. A stock solution of insulin

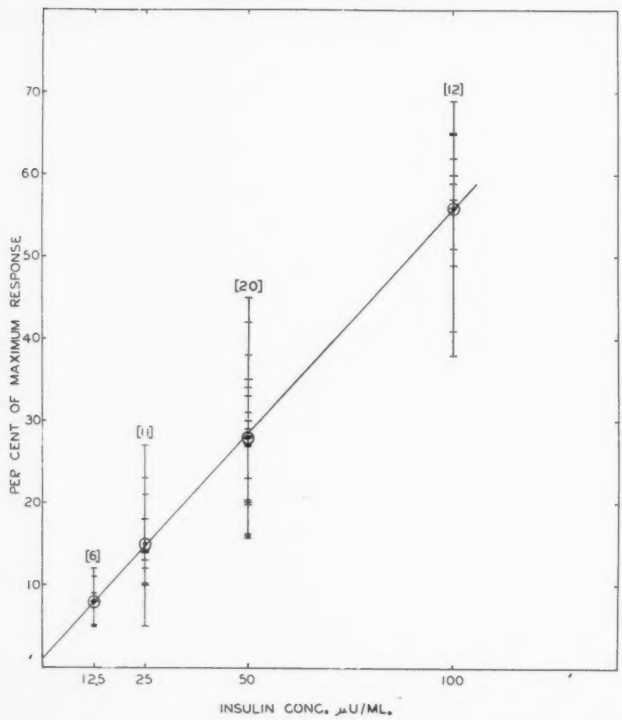

FIG. 2. *The Percentage maximum response of adipose tissue at different concentrations of crystalline insulin.* All experiments were performed in the Krebs-bicarbonate gelatin medium by the procedure described in the text, and as illustrated in Figure 1. The total number of determinations at each insulin concentration is indicated by the figure in parenthesis at the top of each set of values and each determination is represented by a short horizontal line. The average value for each set is represented by a circle.

was prepared by dissolving 6 mg. or 150 units in 5 ml. of water to which 2 drops of 0.1N HCl had been added. This was diluted with the standard bicarbonate-gelatin medium so that 0.05 or 0.10 ml. when tipped from the sidearm yielded a final concentration of 12.5, 25, 50, or 100 micro units (μu) per ml. In making the dilutions a set of 10 ml. glass stoppered graduated glass cylinders have been employed. These cylinders have been reserved for this purpose only and between use are merely rinsed with distilled water, cleaning solution and detergents being avoided. Each time before their use they were rinsed several times with the bicarbonate-gelatin medium. The pipets employed were always rinsed 3 times with the insulin solution which was to be measured out in making the dilution or to be added to the sidearm of the flask. We have found it necessary to pay the strictest attention to details of this sort when working with such small quantities of insulin. In Figure 2, the response of the tissue to these

amounts of insulin is expressed in terms of the percentage of the maximum rate subsequently obtained in the presence of 0.1 unit of insulin per ml. The results presented are the composite of determinations by three different individuals obtained over a period of time. The average value for each set falls on a straight line when plotted directly against the insulin concentration. The slope of this line is 1.8 μ units per percentage point. The only justification to be offered at this time for such a method of plotting is that the calculations are simplified. In this regard it should be noted that in our hands a 95–100% of maximum response is obtained only when the insulin concentration reaches 1,000 μu per ml. Thus over the total dose response range of 12.5 to 1,000 μu per ml. the use of a logarithmic plot would be indicated. However for assay purposes we believe that a greater accuracy may be obtained if concentrations are limited to the range between 15 and 60% of maximum response. When values are obtained outside this range the assay is repeated with amounts which are estimated to fall within this range from the data at hand. For example, when values fall within the range 55–75% of maximum response the solution is best diluted 1:5 for reassay, and 1:10 for values 75–100%. Such a dilution procedure works satisfactorily for the assay of tissue extracts of the sort described in Figure 1 but dilution of serum presents a special problem.

The value of 46% of maximum response obtained for the homogenate of toad fish islet tissue in Figure 1 may now be expressed in terms of insulin equivalents. The response is the same as that given by a solution containing 46×1.8 or 83 μ units of insulin per ml. Since in the assay method the total fluid volume in the flask is 1.5 ml., the total insulin equivalents in 0.05 ml. of toad fish homogenate would be 1.5×83 or 125 μ units. Since this amount of homogenate represents 1 μg of fresh tissue, the insulin-like action of the fresh tissue corresponds to 125 units or 5 mg per gram wet weight. Experiment B in Figure 1 was run in the same manner. In this case the percentage of the maximum response obtained with the human insulinoma homogenate is 29/98 or 30% which yields an insulin equivalence of 54 μu/ml. or 81 μu total in the flask. The 0.05 ml. of insulinoma homogenate that was added at arrow 1 represented 1 μg of fresh tissue. Hence, the insulinoma tissue displays activity equivalent to 81 units of insulin per gram wet weight. In both cases illustrated, the tissue was homogenized in a salt solution buffered at pH 7.4 containing gelatin and dilutions performed with a Krebs HCO_3 salt solution containing gelatin. It should be noted that the results in Figure 1 have been expressed in terms of mm pressure change observed. If it is desired the results can be expressed in terms of gas volume by the use of the appropriate flask constant and in terms of a unit weight of tissue as is done in Figure 3. This is, however, not necessary for assays of the type shown in Figure 1 since it is only relative rates of the reactions which are required, and uncalibrated flasks may be readily employed and needless calculations eliminated.

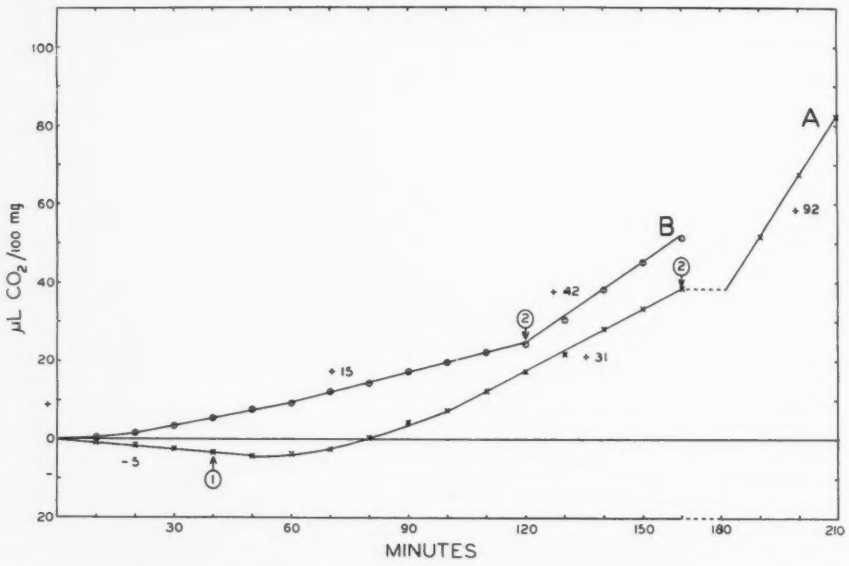

FIG. 3. *An example of the application of the method to normal human serum.* In curve A, 0.1 ml. of serum was tipped in from the sidearm at arrow 1, and 0.05 ml. of a crystalline insulin solution (0.15 μ units) added at arrow 2. In curve B, 1.5 ml. of serum was present from the start and 0.05 ml. of insulin (0.15 μ units) solution tipped in from the sidearm at arrow 2. Rates are indicated in terms of μl of gas per 100 mg. of tissue per hour, and paired tissues from a single rat were employed. Blood was drawn from a normal human male subject (W.E.C.W.) two hours following breakfast, allowed to clot and the serum separated by centrifugation. The serum was fortified with glucose by the addition of 2.6 mg. per ml. The assay was started within one hour after collection of the blood.

SERUM

Though our studies are as yet limited in number, the method has been applied to serum. Here one encounters some puzzling and interesting relationships. Several examples will be described in order to illustrate the pertinent points and to indicate the need for their consideration and further investigation in the application of the method to serum. In Figure 3 an example of the results obtained with normal human serum is presented. One piece of paired tissue (A) was run in the manner already described. Gelatin was not employed in the medium since the high protein content of the serum sample to be added eliminates the need for it. The sidearm of the flask contained 0.1 ml. of the fortified serum and this was tipped in at the time indicated by arrow 1 after the usual initial negative rate had been ascertained. A positive pressure response ensued and reached a fairly linear rate after 60 minutes. The maximum response of the tissue was next obtained by the procedure described and the percentage of maximum response given by the serum calculated to be 37%. The

other piece of paired tissue (B) was placed in a flask containing 1.5 ml. of the fortified serum. As can be seen from Figure 3, this flask showed a positive pressure response from the start of the experiment and the rate of gas release tended to increase during the first hour after which it became nearly linear with time. At the time indicated by the arrow 2, 0.05 ml. of an insulin solution containing 0.15 units was added from the sidearm to obtain the maximum response. The percentage of maximum response in this case is calculated by making the assumption that this piece of tissue would have shown an initial control rate equal to that of the paired piece, namely −5.0. On this basis a figure of 43% is obtained. Thus the serum diluted 1:15 and the undiluted serum yield nearly the same percentage of maximum response. However, expressed in terms of insulin, the activity shown by the undiluted serum corresponds to 79 μ units per ml. as contrasted with a value of 1026 μ units per ml. for the serum diluted 1:15. It should be noted that when undiluted serum is to be run, it is always advisable to run concomitantly a piece of paired tissue in the manner shown here. This not only furnishes a value for the initial rate, but more importantly assures one that any tissue response to the undiluted serum is a specific one.

Several points in connection with the measurement of the insulin like activity of serum by this procedure need comment. First, it may be noted that the maximum response shown by the tissue in undiluted serum (42 μl CO_2 per 100 mg. per hr.) was much lower than that shown by its paired piece in the 1:15 diluted serum (92 μl). This has been a consistent finding. The average difference seen between paired pieces of tissue run in the bicarbonate glucose medium with insulin tipped from the sidearm is of the order of $\pm 10\%$ (5). The same sort of agreement is found if a comparison is made of the maximum response yielded by paired tissue in 1:15 diluted serum and in plain buffer. However, if a comparison is made in plain buffer on paired tissues in which insulin is present from the start in one case and in the other tipped in after an initial incubation period, then the latter always gives a higher response. The reason for this is not known. The difference is however, much less than that observed between undiluted serum and serum diluted 1:15. Hence, other factors would appear to be involved in the case of undiluted serum. Among these may be the CO_2 buffering capacity of the serum protein and the possible presence in serum of substances antagonistic to insulin. It should also be noted that the establishment of a linear response may never be as prompt with serum as with extracts of islet tissues (compare Figs. 1 and 3), or with commercial insulin.

The relative constancy of the percentage of maximum response that is obtained with dilution of normal human serum up to values of 1:15 has been a fairly consistent finding. Table 1 presents some values which illustrate this point. Our experience with dilutions beyond the range of 1:15 is as yet limited. Another point that is illustrated by the data in Table 1 is that when serum is stored in the frozen state and then thawed,

TABLE 1. MEASUREMENTS ON NORMAL HUMAN SERUM

Serum sample	Age of serum	Dilution factor	% maximum response	Insulin equivalents, μu/ml.	Comments
E.G.B.	1 hr.	None	40	72	
	1 hr.	1:2	48	173	
	1 day	1:3	46	248	Stored at 4° C
	1 day	1:6	39	420	Stored at 4° C
	5 days	None	43	77	Stored at −20° C
	5 days	1:15	43	1155	Stored at −20° C
	12 days	1:15	86		Stored at −20° C
R.L.J.	1 hr.	None	47	85	
	1 hr.	1:3	35	189	
	1 hr.	1:15	42	1130	
	4 days	1:15	71		Stored at −20° C
	41 days	None	73		Stored at −20° C
	41 days	1:15	48	1300	Stored at −20° C
H.E.F.	1 hr.	None	63	113	
		1:3	45	243	
		1:15	34	920	
	1 day	1:15	28	755	Stored at −20° C
	36 days	None	43	77	Stored at −20° C
	36 days	1:15	15	405	Stored at −20° C
W.E.C.W.	1 hr.	None	44	79	
		1:3	37	200	
		1:15	38	1025	
C.D.J.	1 hr.	None	60	108	
		1:3	53	286	
		1:15	52	1400	
		1:15	37	1000	Run in gelatin containing medium
		1:75	10	1350	Run in gelatin containing medium
	5 days	None	74		Stored at −20° C
	5 days	1:15	100		Stored at −20° C

All subjects were male. In each case blood was collected approximately 2 hours after the morning meal and allowed to clot. The serum was separated by centrifugation in the cold room and fortified with glucose by the addition of 2.6 mg. per ml. Dilutions were made with the Krebs-bicarbonate medium containing 3.6 mg./ml. of glucose except where noted. In all experiments where the dilution was 1:15 or greater the experiment was run as in curve A of Fig. 3. In all other cases serum was present in the flask from the start of the experiment as in curve B of Fig. 3.

there may be a substantial increase in the percentage of maximum response. This increase may not occur after a single freezing or thawing but require several. At this point it may not be amiss to reemphasize that upon dilution the homogenates of toad fish islet tissue and of human insulinomas displayed a change in activity which was within reasonable proportions to the dilution factor.

DISCUSSION

The method described here is both simple and sensitive. In terms of crystalline insulin it is capable of measuring quantities of the order of 0.01 μg. It should be emphasized that it is not specific for insulin but assays insulin-like material. In unpublished work from this laboratory, it has

been found for example that lactogenic hormone, protamine sulfate, and other compounds yield a response in this test similar to that given by insulin. However, responses comparable to insulin are only obtained at concentrations of these substances at least one hundred thousand times greater. The method probably has the same specificity as that of Renold *et al.* (8) since it measures such substances as will initiate fat synthesis and the concomitant glucose 6 phosphate dehydrogenase pathway in adipose tissue. In addition, it will measure any stimulation of lactic acid production. In this regard it should be noted that both these methods are more specific than those which measure stimulation of glucose uptake by adipose tissue since a number of materials, e.g. growth hormone preparations, adrenaline, glucagon, are capable of stimulating glucose uptake by the tissue without initiating fat synthesis. On the other hand, it may be pointed out that adipose tissue is not without some selectivity in its response. For example, as shown in Figure 1 a good response is obtained with an amount of an homogenate of toad fish islet tissue which represents 1 μg of fresh tissue. However, homogenates of toad fish liver, muscle, kidney, or heart are inactive when tested with amounts equivalent to 2 mg. of fresh tissue. Likewise with homogenates of various rat tissues, only the pancreas shows activity. Human serum albumin is inactive at concentrations up to 5%.

One of the chief advantages of the method is that each piece of tissue serves as its own control and that it is not necessary to run insulin standards concomitantly. This conserves both time, animals, and materials. In addition the method permits one to be certain that the specific piece of tissue to be employed is responding in a normal fashion before the measurement is begun. It would undoubtedly be advisable for each investigator to establish for himself a calibration curve of the type shown in Figure 2.

There is one other advantage that the method possesses which is worth emphasizing. This is the fact that it permits the course of the reaction to be followed at frequent intervals throughout a selected time period so that any irregularity in response is detectable. This advantage is well demonstrated in the measurement of the insulin-like activity of a fraction obtained from human blood by Dr. H. N. Antoniades (9). Antoniades *et al.* (10) have suggested that such a preparation is a complex of insulin with a basic protein. When tested by the rat diaphragm method it shows no insulin-like activity but does so after alkali treatment (11) or as more recently reported (12), after treatment with an adipose tissue extract. When tested by the procedure described here, the preparation[2] showed steadily increasing activity as the incubation proceeded. The tissue showed an

[2] The sample employed, labeled lot #057, was one of those distributed by Dr. Antoniades to those present at the Seminar on the Bioassay of insulin and insulin-like activity held in Los Angeles on January 21, 1960. The results given here were obtained on February 5, 1960, and as agreed upon, were communicated to Dr. Antoniades shortly thereafter.

initial net gas exchange of −3.4 μl per 30 min. The preparation was then added and the net gas exchange values for successive 30 minute periods were as follows: −1.9, −1.4, +3.1, +5.5, +8.8, +11.2, +11.0, +10.8. These results obviously suggest that either adipose tissue responds slowly to this material or that the preparation is gradually altered in the presence of adipose tissue so as to unmask its insulin-like activity. In either case, it is obvious that any assay of its activity is not a straightforward proposition and in any method employing adipose tissue where changes are measured over a fixed time period, the results obtained are likely to vary markedly with the total elapsed time used for the assay.

The data presented here confirm the observations of other workers (13, 14, 15) that a marked increase in the insulin-like activity of serum may occur upon its dilution when values are expressed in terms of absolute units of insulin per ml. of serum. Insulin-like activity corresponding to values well in excess of 1000 μ units per ml. are indicated from the results with diluted serum. If, however, the results are expressed in terms of the percentage maximum response obtained, then there is a remarkable constancy for this value as the serum is diluted up to 1:15. For normal human serum collected about 2 hours after the morning meal this value usually lies within the middle range, 40–60%, of the tissue's maximum ability to utilize glucose. This corresponds to a response that would be elicited by 75–100 μ units of insulin per ml.

Several explanations may be offered to account for such a pattern of response. One may invoke the presence of a bound form of insulin which is dissociated on dilution in such a way as to maintain a uniform effective insulin concentration. Or one may postulate the presence in serum of two substances, one capable of stimulating glucose uptake by the tissue and the other possessing a counteracting effect. The serum thus in a sense, being buffered so that upon dilution the ratio of the two opposing forces would remain constant and hence their relative effective concentrations upon the tissue would be essentially unaltered. A limit to the range within which such dilution could be made would of course depend upon the absolute concentration of the components in the serum in either case. The increase in insulin-like activity upon freezing and thawing of serum would either be explained by a breakdown of the complex or destruction of the anti-insulin factor. A mechanism in which an interplay of two opposing forces occurs has attraction from the standpoint of providing the organism with a smooth control of its rate of utilization of glucose. Though it is tempting in this connection to speculate on the possible interplay of pituitary factors and insulin in such a mechanism this had best await the outcome of experiments in progress.

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FAILURE OF 19-NOR-ANDROSTENOLONE PHENYLPROPIONATE (DURABOLIN) TO PREVENT THE ALTERATIONS PRODUCED BY CORTISONE ON THE GROWING BONE IN RATS

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ABSTRACT

19-nor-androstenolone phenylpropionate and cortisone were given, alone or in combination, to growing rats and effects on body and bone growth were studied.

Under the experimental conditions used Durabolin did not prevent the alterations induced by cortisone.

THE deleterious effects of cortisone on growth as well as on bone and teeth structure have become apparent—both experimentally and clinically—with the increased use of this hormone in recent years (1–6). Some investigators have reported success in counteracting the skeletal alterations produced by cortisone in humans by the administration of androgen hormone (7, 8).

In a previous study with rats (9) it was found that the daily injection of testosterone 0.5 mg. or estrogen 0.05 mg. concomitantly with cortisone 5 mg. daily up to three weeks, did not prevent the effect of the latter on growth and bone.

The present report presents an investigation designated to test the efficiency of 19-nor-androstenolone phenylpropionate (Durabolin), an anabolic preparation, to prevent or ameliorate the effects of cortisone on growth and bone in rats.

MATERIAL AND METHODS

Thirty two male rats of a locally bred Swiss albino strain were used. The rats, kept in separate cages throughout the experiment, were fed regular chow (Purina) and water *ad libitum*. They were divided into four matched groups and treated for two weeks, as shown in Table 1. The rats were weighed at the beginning of the study and one and two weeks after beginning of the treatment. At the end of two weeks the rats were killed by ether. The left tibia was dissected and fixed in formalin. After decalcification with formic acid, a midsagittal cut through the tibial tubercle was made. The specimens were embedded in paraffin and stained with hematoxylin and eosin.

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TABLE 1. GROUPS OF RATS STUDIED

Group	Number of rats	Treatment	Initial weight, in gm. mean ± SD
I	8	none (control)	90 ± 11.4
II	8	Durabolin	92.5 ± 3.5
III	8	Cortisone	93.7 ± 8.5
IV	8	Cortisone & Durabolin	92 ± 3.7

Durabolin = 19-nor-androstenolone phenylpropionate (Organon); daily dose: 2.5 mg.
Cortisone acetate (Adresone-Organon); daily dose: 5 mg.
The drugs were injected by the intraperitoneal route. In Group IV the drugs were injected separately.

RESULTS

The growth as expressed by percentage increment in weight is the different groups studied is shown in Figure 1. It is evident that there are two different growth patterns depending on the administration or non-administration of cortisone. The average size of the rats treated with Durabolin was slightly increased over that of the controls. At the end of the first week the difference between the mean weights was statistically significant ($t = 3$; $P = .01$); at two weeks the difference was not significant ($t = 1.6$; $P = .2$). The rats receiving cortisone lost weight during the first week, but subsequently responded unequally; some gained weight whereas the others continued to lose weight. The rats treated with cortisone and Durabolin concomitantly showed the same growth pattern as those treated with cortisone only. Though the mean weight of the cortisone and

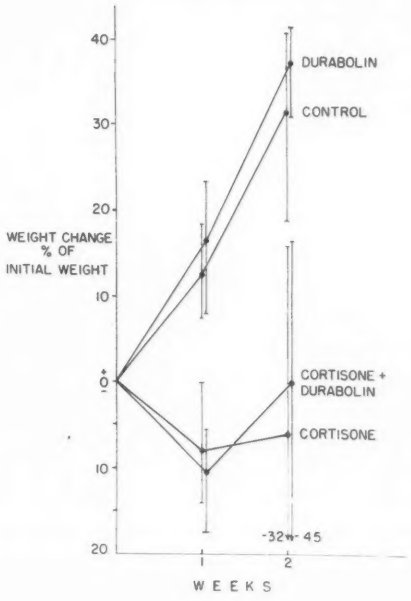

FIG. 1. Growth of rats, untreated control and treated groups, expressed as percentage change in body weight (mean ± range).

Durabolin-treated rats was greater than that of those treated with cortisone alone at the end of the second week, the difference was statistically not significant ($t = 0.59$; $P = .6$).

The histological appearance of the proximal rat tibia in the groups studied is illustrated in Figure 2. In Figure 2-A the normal epiphysis and metaphysis are seen. The structure of the tibia in the Durabolin treated group is essentially similar to that of the control animals; however, in some rats the width of the epiphyseal cartilage is slightly increased (Fig. 2-B). The tibiae of the rats treated with cortisone (Fig. 2-C) reveal a marked reduction in the width of the epiphyseal cartilage involving both the proliferative and hypertrophic zones. The epiphyseal-metaphyseal border is irregular. The trabeculae of the primary spongiosa are of irregular shape and contain an increased amount of provisionally calcified cartilagenous matrix. There is marked reduction to patchy absence of the connective tissue penetrating the cartilage; correspondingly, the number of osteoblasts is reduced and osteoid seams are scant. The width of the primary spongiosa and the number of trabeculae is reduced. In most rats of this group the fat tissue of the bone marrow is increased.

When Durabolin was given concomitantly with cortisone (Fig. 2-D) the histologic changes strongly resemble that of Group C. Thus, the epiphyseal cartilage is thinned; the epiphyeal-metaphyseal border is irregular, though to a lesser degree than in the cortisone group in some of the rats. The primary spongiosa resembles either the cortisone group or the changes were more marked as illustrated in the figure. In two rats the changes were of a lesser degree. It is noteworthy that in sharp contrast to the scant penetrating connective tissue in the cortisone group, there is comparatively abundant penetrating connective tissue in the cortisone and Durabolin treated rats.

COMMENTS

The reports on the efficacy of androgenic hormones to counteract the catabolic effects of cortisone and its derivatives are conflicting. In the present investigation none of the deleterious effects produced by cortisone on animal growth and bone structure were prevented or diminished by the administration of Durabolin. Similar negative results have been obtained using testosterone alone or in combination with estrogen (9). Frieden *et al.* found testosterone and some of its derivatives unable to diminish the inhibitory effect of cortisone on the amino-acid incorporation in mouse kidney homogenate (10). Of special interest is the report of Kowalewski who studied the radiosulfate uptake in growing bones of cockerels (11). While methyltestosterone and testosterone propionate were unable to counteract the anti-anabolic effect of cortisone, 17-ethyl-19-nortestosterone attained the effect. In later reports Kowalewski and Gort (12) and Grossman *et al.* (13) claimed similar results in rats.

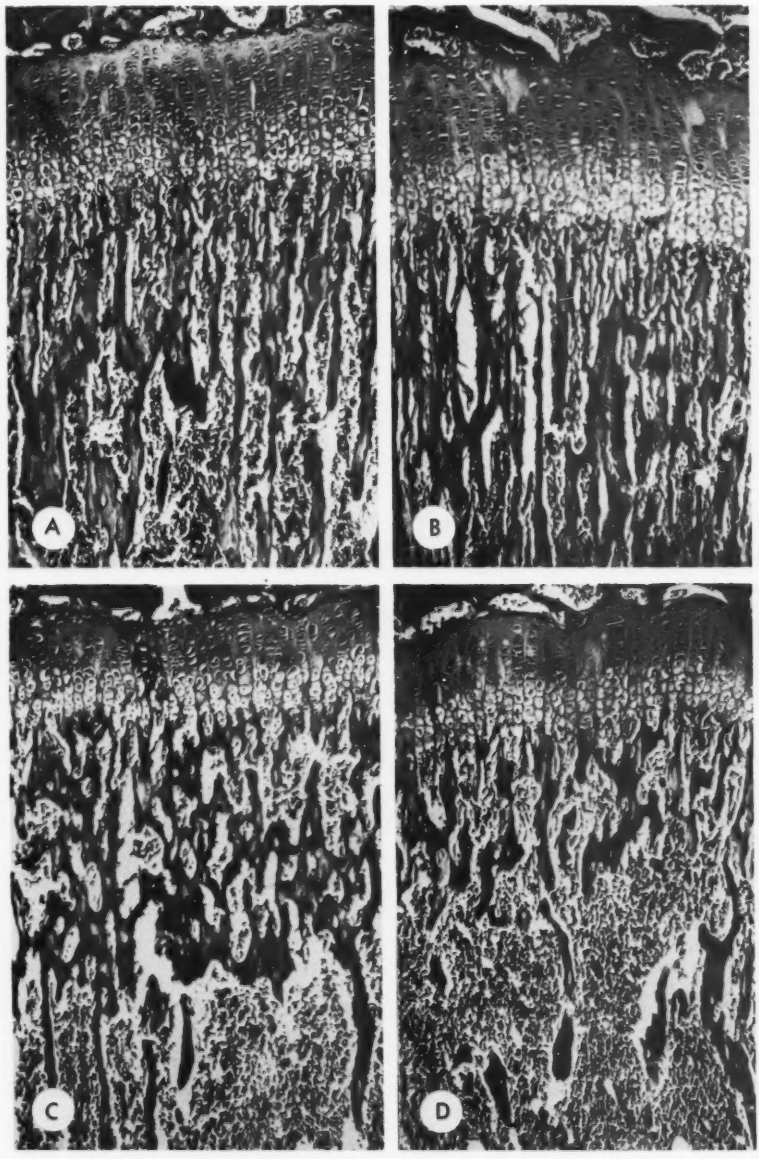

FIG. 2. Histological appearance of the proximal epiphysis and metaphysis of the rat tibia. A—control; B—Durabolin 2.5 mg.; C—Cortisone 5 mg.; D—Durabolin 2.5 mg. and Cortisone 5 mg. Hematoxylin and Eosin ×40.

Observations in adults showed that androgens, including testosterone and estrogens may counteract the harmful effects of cortisone on calcium and nitrogen metabolism (7, 8). It should be stressed that in these studies bone biopsies were not performed. If our previous (9) and present findings in growing rats will hold true for children receiving long term treatment with cortisone or its derivatives, by analogy the addition of testosterone or the newer less virilising anabolic substances would not restore normal growth or heal the osseous lesions and would only add the harmful effects of these hormones. It should be noted, however, that the dosage per body weight of both cortisone and androgenic or anabolic substances used in the previous and the present animal experiments are high compared to the dosage per body weight used in humans. Therefore final judgement as to the effect of anabolic drugs in counteracting cortisone action in humans should be withheld until further studies using different anabolic/cortisone ratios have been carried out.

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CONVERSION OF ANDROSTENEDIONE-4-C^{14} TO ESTRONE BY THE MARMOSET PLACENTA[1]

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ABSTRACT

Conversion of androstenedione-4-C^{14} to estrone has been reported using a preparation of the marmoset monkey placenta *in vitro*. A possible relationship of placental endocrine function with fetal sexual development as it might pertain to the free-martin has also been discussed.

ALTHOUGH there is a considerable body of biological data correlating endocrine function of the placenta with viviparity (1), there is still a paucity of direct information on hormone production by this organ in species other than man. Aromatization, an important step in estrogen formation, has been well documented in the human placenta (2), but not in the placentas of other animals.

The present report is part of a study of the comparative biochemistry of the placentas of various species and documents the conversion of androstenedione-4-C^{14} to estrone by the marmoset monkey placenta in approximately 10% yield. The rationale for the use of this particular species to initiate the study came from a consideration of the possible relationships of placental function and structure to the etiology of the Free-martin. Free-martins occur in cattle pregnancies with relative frequency, and since Lillie's classic observations in 1917, the best theory relating to their development is still the concept of *in utero* sterilization of the female cattle fetuses by their male twin partners, presumably by a blood-borne androgen (3). This is possible only by virtue of the vascular anastomoses between fraternal twin placentas in cattle. Although such vascular anastomoses are rarely found in other species, they do occur in marmosets (4) and man (5). However, the Free-martin has not been observed in human

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or marmoset pregnancies. While the etiology of the Free-martin is still obscure, it is possible that special enzymatic properties of the placenta may be the reason for the species differences.

EXPERIMENTAL

Marmoset Pregnancy: A pair of "lion marmosets" (Leontocebus rosalia) was purchased shortly after import and kept in a large cage at 85–95° F. Their maintenance diet consisted of fruit, milk, meal worms and bread with occasional supplements of ground fresh beef and newborn rodents which are particularly important during pregnancy. Ultraviolet exposure was given daily for 15 minutes. Approximately two months after capture, it became apparent from an increase in girth and weight that the female was

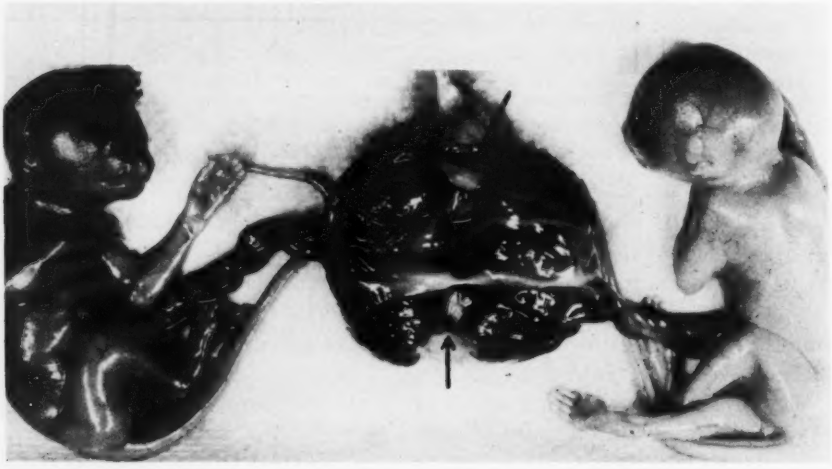

FIG. 1. Photograph of twin marmoset placenta and fetuses. Prominent vascular anastomoses are indicated by arrows.

pregnant. At approximately four months of gestation, the female was anesthetized with an intraperitoneal injection of 0.3 ml. veterinary Nembutal and laparotomy performed 45 minutes later. A typical twin pregnancy was interrupted. The two female fetuses were alive, each weighing 26.5 gm. and measuring 8.4 cm. crown to rump and 17 cm. snout to tail. The placenta was a fused organ with at least two prominent vascular anastomoses (Fig. 1). It weighed 17.8 gm. and measured $3\times3\times0.5$ cm.

Tissue preparation and Incubation: The placental tissue was prepared as previously described for the human placenta (2) and the $800\times$gm. supernatant fraction was employed. A portion of this preparation equivalent to 8.2 gm. wet weight of tissue was incubated with 13.2 μg androst-4-ene-3,17-dione-4-C^{14} (1.3×10^6 cpm.) (New England Nuclear) in phosphate buffer at pH 6.8 with 10 μM ATP and 10 μM DPN in a total volume of 5 ml. at 37° C for four hours with air as the gas phase. A zero time control flask containing the entire system was prepared and processed in parallel with the incubated flask.

Measurement of Radioactivity: Determination of radioactivity was carried out by plating at infinite thinness into disposable aluminum planchets and counting in a gas-flow counter operated in the Geiger region. The procedure employed by the Biophysics Department of Harvard Medical School was followed (6).

Estrogen Measurements: Analyses of estrogens were carried out in duplicate using the sulfuric acid fluorescence technique described by Slaunwhite *et al.* (7).

Column partition chromatography was employed using a Celite column prepared as described by Engel *et al.* (8). The formation of estrone methyl ether and its subsequent purification and alumina chromatography were performed as described by Brown (9). Paper chromatography was carried out using the Bush method (10) at room temperature with solvent systems noted in the text. Details of chromatography and elution have been previously reported (11).

RESULTS

Initial purification: The contents of the incubation flask and the zero-time control were extracted 3 times with 6 volumes of chloroform and processed individually. The aqueous phase and tissue were treated with ten volumes of hot acetone, the mixture filtered and the acetone evaporated. The remaining aqueous residue was then re-extracted with 6 volumes of chloroform 3 times and the chloroform fractions pooled and evaporated to dryness in vacuo. The recovery of radioactivity to this point was 85%. The chloroform residue was then subjected to a phenolic purification between toluene and 1N NaOH (12). In the incubated experiment, 25% of the starting radioactivity (33, 488 cpm) was recovered in the phenolic fraction. The zero time control contained less than 1% of the starting radioactivity in this fraction and was not processed further.

Purification of the Phenolic Fraction: The phenolic fraction containing 33,488 counts per minute was next subjected to an eight transfer counter-current distribution between toluene and N NaOH (13). The higher numbered tubes which contained the phenolic fraction accounted for 70% of the applied radioactivity (22, 347 cpm) (Fig. 2). Tubes 5–8 of the distribution were pooled and applied to a Celite column with 200 μg each of estrone and estriol and 220 μg of estradiol as carriers. Alternate tubes off the

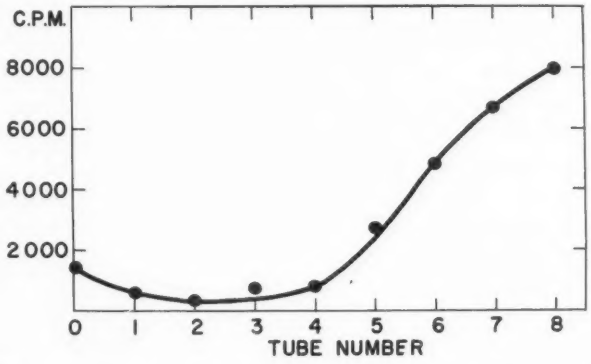

FIG. 2. Eight transfer counter current distribution between toluene and 1 N NaOH. The lower layers were transferred. The phenolic fraction is contained in the higher numbered tubes.

column were counted and in the areas of concentration of the counts, each tube was counted for radioactivity and analyzed for estrogens by fluorescence in duplicate. Ninety-seven percent of the applied radioactivity was recovered off the column. The peak for radioactivity and for estrone carrier fluorescence coincided and the curves for both counts per minute and carrier fluorescence were superimposable (Fig. 3). The estrone curve contained 39% of the applied radioactivity and the recovery of the estrone carrier was 90%. After the estrone had been recovered from the column, gradient elution was started employing ethylene dichloride:trimethyl-

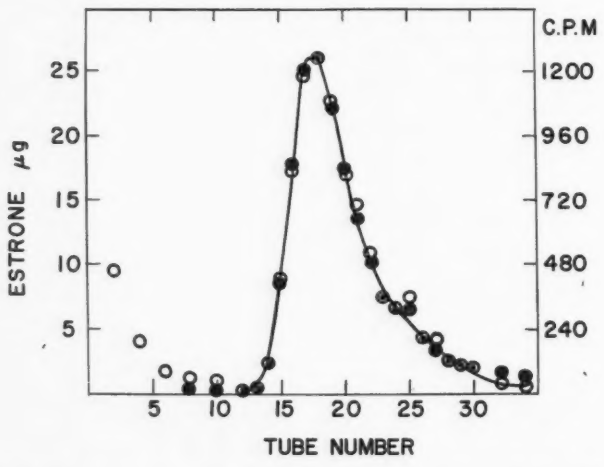

FIG. 3. Partition chromatography of the phenolic fraction on Celite. The stationary phase was 90% methanol, the moving phase was trimethylpentane. The closed circles represent fluorescence of estrone carrier, the open circles represent cpm.

pentane (1:1) and 96 tubes of one column volume each were collected. There was a peak of radioactivity in the estradiol portion of the curve which coincided with the estradiol carrier and accounted for 9% of the applied radioactivity. In addition, there were peaks of radioactivity on either side of the estradiol, but these metabolites have not been identified. There was no radioactive peak in the estriol portion of the curve and the carrier estriol was recovered devoid of radioactivity.

Purification of Radioactive Estrone: Statistical analysis of the estrone curve off the Celite column (Fig. 3) revealed a specific activity of 50.3 ± 3.9 cpm/μg with a Chi-square of 4.5 and a P of 0.9 over 16 tubes. The tubes under the curve were pooled and aliquots subjected to paper chromatography in 3 systems as described in Table 1. The radioactivity coincided with the carrier estrone in each system and the specific activity was constant (Table 1). The estrone eluted from the chromatograms was pooled,

TABLE 1. PURIFICATION OF RADIOACTIVE ESTRONE

Purification step	C.P.M.	μg.	Specific activity μg/cpm
Celite Column (16 tubes)			50.3 ± 3.9
Paper chromatography*			
Ligroin/96% methanol	980	18.9	51.9
Ligroin:toluene (2:1)/70% methanol	1298	25.5	50.9
	1476	28.3	52.2
Toluene/75% methanol	1584	30.3	52.3
Methylation	4495	95	47.3
Alumina chromatography	4155	88.5	46.9
Paper chromatography* of methyl ether			
Ligroin/96% methanol	862	17.8	48.4

* Aliquots of the total pool were used for paper chromatograms.

methylated and purified by solvent partition and alumina chromatography (9). An aliquot of the methyl ether was also subjected to paper chromatography after purification on alumina. Through all these steps the specific activity remained essentially constant (Table 1).

In a separate experiment with another marmoset placenta where purification was carried only through Celite partition chromatography, similar results were obtained.

DISCUSSION

The conversion of androstenedione-4-C^{14} to estrone has now been demonstrated for the marmoset placenta in a manner analogous to studies with human material. Radioactive substrate was employed in these experiments because of the limited supply of tissue and the unpredictability of the extent of conversion. It is apparent however that the marmoset placenta is active in the aromatization reaction.

The criteria for radiochemical purity of the radioactive estrone employed in this study are similar to those proposed by Baggett *et al.* (14). An eight transfer distribution was employed to separate the phenolic fraction, and the fine resolution of Celite partition chromatography was employed with statistical evaluation of the specific activities of the individual tubes in the estrone curve. Subsequent purification on multiple paper chromatograms and the relatively specific procedures of Brown's methyl ether formation and alumina chromatography (9) were utilized with a constant specific activity of the estrone through all the purification steps.

Although it is reasonable to assume that this aromatization reaction is part of the normal biosynthetic pathway for estrogen production, this point still needs rigorous confirmation. From a comparative point of view, it would be interesting to see if the placentas of all species capable of placental estrogen formation contain this enzyme system, and conversely to check for its absence in the nonestrogen producing placenta. Pertinent to this study are the preliminary unpublished data with cattle placentas processed in a manner similar to that described previously (2). Using an

incubation system as reported for the human placenta, aromatization could not be demonstrated. It is planned to repeat this work with radioactive substrates. The initial negative results may well be due to technical limitations, but the cow cannot maintain pregnancy after oophorectomy without some replacement therapy, suggesting that the cow placenta does not produce as much steroid hormone as the placentas of other species (*cf.* 1).

The relationship of the aromatization reaction to the Free-martin is quite immediate. In cattle, the occurrence of the Free-martin in fraternal twin pregnancies may be due to an "androgen" produced by the male fetus which exerts its effect via vascular anastomoses between the twin placentae. Such an "androgen" might not be expected to persist or accumulate in the presence of a highly effective aromatizing enzyme system. This may account for the absence of the Free-martin in human and marmoset pregnancies, in spite of known vascular anastomoses, which in the human are sufficient to result in blood group chimeras (4, 5).

Acknowledgment

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A RAPID, SENSITIVE ASSAY PROCEDURE FOR ADRENAL STEROID-3β-OL-DEHYDROGENASE ACTIVITY[1,2]

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ABSTRACT

A sensitive, rapid method has been standardized for measurement of steroid-3β-ol-dehydrogenase activity in the adrenal gland by determining the amount of ultraviolet absorbing (240 mμ) material formed from dehydro*epi*androsterone. The method permits assay of the activity of single adrenals of individual rats, thus making it possible to determine activity by both biochemical and histochemical techniques in individual experimental animals. Activities of bovine, rabbit, and human adrenals have also been determined.

THE enzyme steroid-3β-ol-dehydrogenase can be demonstrated by biochemical and histochemical means in all normal steroid-hormone-producing tissues (1–6), but is not detectable in other tissues. This enzyme converts C_{19} and C_{21} steroids with the Δ^5-3β-hydroxyl structure to those with the Δ^4-3-ketone grouping characteristic of most of the active steroid hormones. It apparently differs from an enzyme present in liver that catalyzes the reduction of 3-keto-steroids (7–9) and which has been named 3β-hydroxysteroid dehydrogenase.

To correlate histochemical observations with biochemically measured differences in function of a steroid-producing gland, it is desirable to study the enzyme by both types of method in tissue from individual animals. A biochemical technique has now been devised that is sufficiently sensitive to permit determination of steroid-3β-ol-dehydrogenase activity in a single rat adrenal. It is thus much more sensitive than a method previously published (3), which required 3–5 gm. of bovine adrenal, or the combined adrenals of five or more guinea pigs or of approximately 50 rats. It is also more rapid and more easily performed.

MATERIALS AND METHODS

Sprague-Dawley rats (Charles River Farms) were killed by decapitation and exsanguination. The adrenals were weighed, then immediately homogenized or frozen.

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[1] Supported by Research Grant A-2795, National Institutes of Health, United States Public Health Service.

[2] Langer and Podos (*Prog. 43rd Endocrine Soc. Meet.*, p. 48, 1961) have suggested the more specific name Δ^5-3β-hydroxysteroid dehydrogenase for this enzyme.

Bovine adrenals were obtained at the slaughter house and frozen as soon as possible. Rabbit adrenals and a normal human adrenal[3] were also frozen. Acetone-dried preparations of beef and rat adrenals were made by homogenizing the tissue in a Waring blendor in the cold two times with ten volumes of acetone each, followed by drying overnight at 4° C over P_2O_5. The powders were stored at −15° C.

The tissue-homogenizing medium consisted of equal parts of 0.9% sodium chloride and 0.1 M phosphate buffer, pH 7.4 (3). On the basis of previous experience (3), and depending on the amount of tissue available, the final volumes of homogenate were adjusted so that the tissue/volume relationships in the "concentrated" homogenates were as follows: rat adrenals, 2–5 mg./ml.; rabbit adrenal, 66 mg./ml.; human adrenal, 200 mg./ml.; bovine adrenals, 350–700 mg./ml. According to the amount of material available, incubations were carried out with two to five different concentrations of each homogenate, dilutions being made with phosphate-saline.

The tissue preparations were incubated in a Dubnoff shaking incubator in air, at 37° C, in 25-ml. Erlenmeyer flasks with ground-glass necks. For each tissue concentration, four flasks were used. Two flasks were tissue blanks and contained 1.6 ml. of homogenate, 0.1 ml. propylene glycol, and 0.2 ml. of a solution of diphosphopyridine nucleotide (DPN) containing 30 mg./ml. Two flasks contained the above materials plus 500 μg dehydro*epi*androsterone.[4] In some experiments, Δ^5-pregnen-3β-ol-20-one was substituted for dehydro*epi*androsterone.

Two extraction procedures have been used. In early studies (procedure A), after incubation, 0.10 ml. 3 M acetate buffer, pH 5, was added to the flasks to stop the reaction. Ten ml. of ethyl acetate were added and the flasks were stoppered, shaken vigorously by hand 50 times, and allowed to stand overnight at room temperature. The ethyl acetate was decanted and dried by addition of sodium sulfate. In later studies (procedure B), the reaction was stopped by plunging the flasks into ice and adding 0.2 ml. glacial acetic acid. After addition of 10 ml. of ethyl acetate, the flasks were shaken 50 times by hand and then placed on an A. H. Thomas rotary shaker, at 4° C, for a half hour. The ethyl acetate layer was then decanted and allowed to stand overnight at room temperature, over sodium sulfate.

Following either extraction procedure, aliquots were evaporated to dryness, the residue was dissolved in ethanol, and the absorption at 240 mμ due to the Δ^4-3-ketone grouping determined in a Beckman D.U. spectrophotometer. The excess ultraviolet-absorbing material formed in the presence of dehydro*epi*androsterone was considered to represent Δ^4-androstene-3,17-dione. (See next section.) When the substrate was Δ^5-pregnen-3β-ol-20-one, the material formed was considered to be progesterone. Results are reported as μg. Δ^4-androstenedione formed per minute per mg. nitrogen. Nitrogen content of the homogenates was determined by a micro-Kjeldahl procedure.

EXPERIMENTAL

1. *Validity of analysis for Δ^4-androstene-3,17-dione by ultraviolet absorption.* Homogenates of bovine adrenals and pooled adrenals of rats were incubated in amounts large enough to catalyze conversion of dehydro*epi*androsterone into quantities of Δ^4-androstenedione that could be isolated by paper chromatography and measured by the Zimmermann reaction.

[3] Removed at operation for carcinoma of the kidney and obtained through Dr. Joseph W. Jailer and Dr. Donald A. Holub of the Department of Medicine, College of Physicians and Surgeons, Columbia University.

[4] The dehydroepiandrosterone was generously provided by Dr. A. D. Odell of General Mills, Inc.

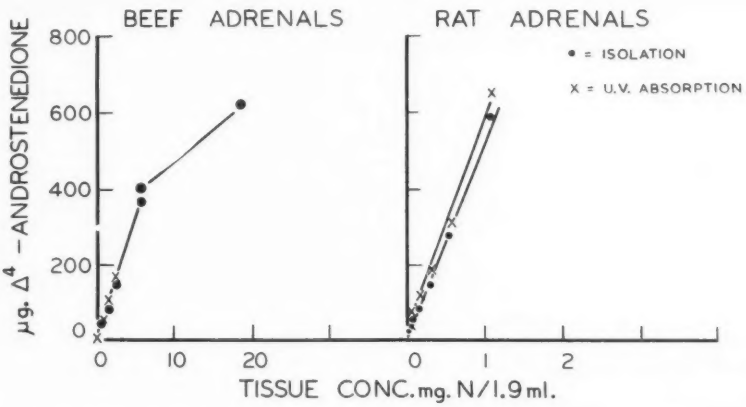

FIG. 1. Validity of analysis by ultraviolet absorption: comparison of results obtained by ultraviolet absorption (at 240 mμ) with results obtained by isolation of product in two typical experiments. These experiments are in terms of tissue concentration.

Ethyl acetate extracts were made as described in (3), and Δ^4-androstenedione was measured in two aliquots, by the isolation procedure (3) and the present spectrophotometric method. As can be seen in Figure 1, curves obtained by the two methods were the same within experimental error.

In addition, as shown in Figure 2, the ultraviolet absorbing material

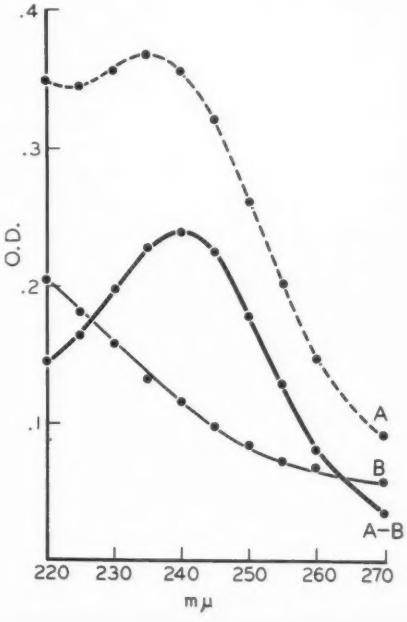

FIG. 2. Spectra of ethyl acetate extract from a rat adrenal homogenate: A = spectrum of extract from flask incubated with substrate, B = spectrum of endogenous absorbers in extract from tissue blank, A–B = spectrum of product formed from substrate upon incubation.

found in excess of that of the tissue blank after incubation of rat adrenal homogenates with substrate had a well-defined absorption maximum at 240 mμ, which agrees with the absorption maximum for Δ^4-androstenedione.

Recovery of added Δ^4-androstenedione, using procedure B, was $97 \pm 2.2\%$.

2. *Optimal assay conditions.* Incubations of bovine adrenal homogenates for periods from 0 to 80 minutes (Fig. 3), in which the extraction was by procedure A, showed that the rate of Δ^4-androstenedione production was linear over the entire period, but the curve did not pass through the origin. The same was true with rat adrenals. With procedure B (Fig. 4), the production of Δ^4-androstenedione by rat adrenal preparations was linear with time over 60 minutes in one experiment and over 30 minutes in another and the curves did pass through the origin. A 30-minute incubation period was chosen for the standard procedure.

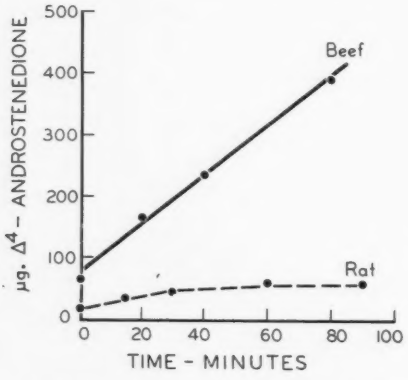

FIG. 3. Production of Δ^4-androstenedione by bovine and rat adrenal homogenates, as a function of time (procedure A).

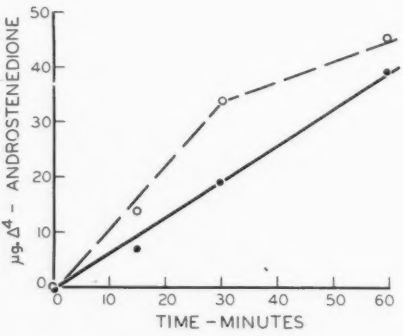

FIG. 4. Production of Δ^4-androstenedione by rat adrenal homogenates, as a function of time (procedure B).

Optimal conditions for obtaining linearity with time have not yet been found for such steroid-producing tissues as testis, ovary, and placenta, although 3β-ol-dehydrogenase activity can be demonstrated in all these tissues by the biochemical procedure, as well as histochemically.

The optimum range of tissue concentration was determined for rat adrenals. Figure 5 shows that the amount of product formed is proportional to the tissue concentration only at very low tissue levels (less than 0.2 mg. nitrogen/1.9 ml.). Previously (3), a linear relation was found for bovine adrenal homogenates over the range from 1 to 10 mg. nitrogen (see also Fig. 1). With tissues of other species, when limited amounts are available and optimum tissue concentrations cannot be predetermined, it is

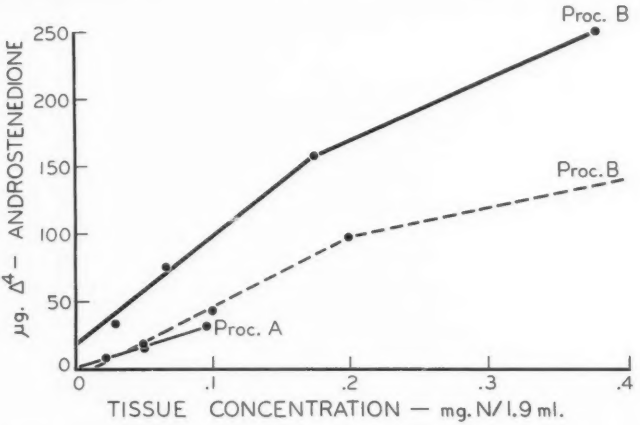

FIG. 5. Production of Δ^4-androstenedione by rat adrenal homogenates, as a function of tissue concentration (30-minute incubations).

best to measure activity by assaying at several tissue concentrations and using that portion of the curve that is linear with tissue concentration and which most nearly passes through the origin.

Dehydro*epi*androsterone was a better substrate than Δ^5-pregnen-3β-ol-20-one for the enzyme in rat adrenals. Two and a half times as much product was formed from dehydro*epi*androsterone per minute per mg. nitrogen. A range of concentrations of dehydro*epi*androsterone from 125 μg to 1 mg. was tested, and 500 μg dehydro*epi*androsterone per 1.9 ml. was judged optimal and adopted for the standard procedure.

Optimal DPN concentration was determined to be 6 mg./1.9 ml. The range tested was 1.5 to 12 mg./1.9 ml. This was true for large amounts of tissue (bovine) as well as for small (rat).

RESULTS

1. *Demonstration that adrenals of a single animal are equal in activity.* The activities of the individual adrenals of two adult male rats were determined individually (Table 1). In each the activities of the left and right adrenals agreed within 10%, and assay of either gland would demonstrate the difference between the animals.

TABLE 1. COMPARISON OF ACTIVITIES OF LEFT AND RIGHT ADRENALS OF ONE ANIMAL PER EXPERIMENT

Procedure	Gland	Wt. of gland, mg.	μg product/min./mg.N
A	left	37	19.8
	right	34	22.6
B	left	33	11.4
	right	32	9.2

TABLE 2. STEROID 3β-OL-DEHYDROGENASE ACTIVITIES OF ADRENALS OF VARIOUS SPECIES

Species	Pooled glands (P) or glands from individuals (I)	Condition of tissue	Extraction procedure	No. preparations assayed	μg Δ^4-androstenedione /min./mg.N (Mean ± S.E.)
Rat, male	I	Fresh	A	9	16.0 ± 2.8
			B	9	14.4 ± 1.2
	P	Frozen	A	8	11.0 ± 1.2
		Acetone-dried	A	2	0.40
Rat, female	I	Fresh	A	1	17.2
Ox	P	Frozen	A	7	1.14 ± 0.14
		Acetone-dried	A	7	0.61 ± 0.10
Rabbit, female	I	Frozen	A	1	0.33
Human	I	Frozen	A	1	3.9

2. *Steroid-3β-ol-dehydrogenase activities of adrenals of various species.* Table 2 lists the adrenal activities in various species.

Fresh adrenals from adult male and female rats had equal activities, forming on the average 15 μg of Δ^4-androstenedione per minute per mg. nitrogen. Freezing did not decrease activity significantly. However, an acetone-dried powder had only about 2.5% of the activity of fresh tissue. There was no significant difference between values obtained with procedure A and those obtained with procedure B.

Frozen bovine adrenals had an enzyme content catalyzing transformation of about 1.2 μg of dehydro*epi*androsterone to Δ^4-androstenedione per minute per mg. nitrogen—approximately 10% of the activity of the rat adrenal. The activity of the acetone-dried powder was about one-half this value. Frozen rabbit adrenals had about 3% of the activity of rat adrenals. A normal human adrenal had an activity intermediate between that of rat and bovine adrenals.

DISCUSSION

The method described is easily performed and is sensitive enough to assay the steroid-3β-ol-dehydrogenase activity in a single rat adrenal. Since the activities of the two adrenals are equal, one gland can be used for biochemical observation and the other for histochemical observation.

The activities in the adrenals of various species of animals measured by this procedure cannot be compared with those reported (1–3), because, on the basis of data now available, the conditions of assay used in those earlier studies were not optimal with regard to duration of incubation and perhaps not optimal for all tissues with regard to tissue concentration.

The secretory activity of the adrenal glands of all these various species has not been determined as yet by comparable methods. However, Vogt

(10) found that the output of corticosterone by rat adrenals was about 80 μg per gland per kg. body weight per hour, whereas that of rabbit adrenals was 30 μg. Possibly one rate-limiting factor in this difference lies in the different degrees of activity of steroid-3β-ol dehydrogenase.

The assay procedure cannot as yet be used for quantitative measure of the enzyme activity in other steroid-hormone-producing tissues, although it can demonstrate the presence or absence of activity. Requirements for optimal activity with testis, ovary, and placenta are under investigation. Perhaps the differences in requirements in those tissues reflect further conversions of Δ^4-androstenedione to compounds no longer absorbing at 240 mμ, e.g., an estrogen. In the adrenal also, the leveling off at higher tissue concentrations might be a reflection of reactions removing Δ^4-androstenedione as it is formed. The addition of larger amounts of substrate or of DPN was without effect in any of the tissues.

More probably the differences in requirements reflect differences in the relative concentrations of steroid-producing cells in the various organs. Histochemically, we have demonstrated (5) that all adrenocortical cells are active, whereas only the Leydig cells of the testis are active, and much of the stroma and the follicular epithelium in the ovary are inactive. And in the rat's placenta, only a small portion of the trophoblast is active (6). In the testis, placenta, and to a lesser degree in ovary, there is, therefore, a significant amount of inactive tissue to dilute the active cells. Other enzyme systems in the inactive tissues may use up endogenous co-factors as yet undetermined, or the inactive tissue may produce inhibitory substances.

Acknowledgments

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PARTIAL DEIODINATION OF L-THYROXINE[1,2]

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ABSTRACT

The unidentified glucuronide previously described in dog bile or in urine of the dehepatized dog following the administration of I^{131}-thyroxine (T4) has been identified chromatographically as 3,3′,5′ triiodothyronine glucuronide. 3,3′,5′ Triiodothyronine (3,3′,5′ T3) also was found in smaller amounts in the free state in bile and in the plasma of the dehepatized dog. Some of this compound was conjugated with sulfate, and some was found in an unidentified acid-labile conjugate. Formation of 3,3′,5′ T3 from thyroxine (T4) involves removal of an iodine atom from the phenolic ring, and thus is the same type of reaction noted earlier in the conversion of 3,5,3′ triiodothyronine (T3) to 3,3′ diiodothyronine. Loss of this iodine atom results in biologic inactivation of these thyroid hormones.

Another metabolite of T4 was found to be 3,3′ T2 which was conjugated with sulfate. Formation of this substance involves the removal of one iodine atom from each of the rings. In spite of this evidence for removal of I^{131} from the ring with the alanine side chain by extrahepatic tissues, deiodination of this ring of T4 is much less complete in dehepatized dogs than in dogs with livers as shown by the decreased output of radioactive iodide in the urine of the dehepatized dog.

A PARTIALLY deiodinated radioactive metabolite of 3,5,3′ L-triiodothyronine (T3), labeled with I^{131} in position 3′, namely 3,3′ diiodothyronine (3,3′ T2), has been found as a sulfoconjugate in the bile of the dog (1). Our studies have indicated that more 3,3′ T2 sulfate than T3 sulfate is excreted in bile and that as much I^{131} is excreted in bile in these two sulfoconjugates as in T3 glucuronide (2). 3,3′ T2 sulfate is readily formed from T3 by the dehepatized dog also, and large amounts are found in both the blood (3) and urine (4). This sulfate plus smaller amounts of T3 sulfate and the sulfate of an unidentified compound accounts for most of the I^{131} excreted in the urine of the dehepatized dog.

In earlier studies Roche and associates (4) and Closon (5) identified T3 sulfate in bile of the rat after the injection of T3. Trace amounts of free

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[2] This investigation was supported in part by Research Grant A-2780 from the National Institute of Arthritis and Metabolic Diseases, Public Health Service.

3,3′ T2 also have been found in the kidney (6) and muscle (7) of the rat after the injection of T3.

Removal of iodine from position 5 in either thyroxine (T4) or T3 results in calorigenically inactive compounds, namely 3,3′,5′ triiodothyronine (3,3′,5′ T3) or 3,3′ T2 (8, 9). Because we found 3,3′ T2 in large amounts as a metabolite of T3, chiefly in conjugated form, we looked for 3,3′,5′ T3 in the conjugates excreted in bile of the dog given T4 and also in blood and urine after hepatectomy. We previously described an unidentified glucuronide which accompanied T4 glucuronide in dog bile (10) and in the urine of the dehepatized dog (11); from this a compound was liberated on incubation with β-glucuronidase which had Rf's on paper chromatograms just below those of T4. We also looked for sulfoconjugates of T4 or its derivatives.

METHODS

Dogs weighing from 6.0 to 12.0 kg. were used in this study. L-T4 labeled with I^{131} in the 3′ or 5′ position was given by single intravenous injection in doses of 28 to 63 μc or 1.1 to 4.9 μg per kg. of body weight. Injections were given to six dogs half an hour to 2 hours after total hepatectomy (12), and to five dogs each with intact liver after cannulation of the common bile duct and cholecystectomy. In five other dogs with intact liver, T4 was injected 1 to 2 days after the biliary fistula was established. Saline solution or a mixture of saline and dextran solution was given by continuous intravenous injection at the rate of 20 ml. per hour to the dehepatized dogs. Glucose dissolved in this solution was given at the rate of 150 mg. per kg. per hour for 6 to 15 hours and then at higher rates up to 350 mg. when indicated. Saline solution was injected intravenously into all but two of the dogs with biliary fistulas at the rate of 20 ml. per hour.

Bile and urine from biliary fistula dogs and urine from dehepatized dogs were collected continuously throughout the experiment, and blood was collected at intervals. The radioactivity of bile, urine and plasma was measured in a well-type NaI (thallium activated) scintillation counter. Column and paper chromatography were used to separate the iodine compounds. The enzymes, bacterial β-glucuronidase and Mylase P, were used to hydrolyze the I^{131} compounds which were conjugated with glucuronide and sulfate respectively (1).

Extracts of urine, bile and plasma were made with four volumes of 1:1 methanol-acetone, as previously described (1). Dried methanol-acetone extracts were dissolved in 50% ethanol. For the chromatograms, aliquots containing approximately 0.03 μc of I^{131} were applied on a spot near one corner of 10 by 12-inch sheets of Whatman 3-MM filter paper and aliquots of 0.6 μc were streaked on 1½ by 22-inch strips of the same paper. The two-dimensional chromatograms were developed in butanol-dioxane-ammonia (BDA) for 20 hours and then in tertiary amyl alcohol, 2N NH_4OH (TAA), for 2 days. Radioautograms of the chromatograms were made on "no-screen" x-ray film. The iodide marker was located with palladium chloride; the thyronine markers, with diazotized sulfanilic acid. Markers run with the unknowns on the chromatograms included iodide, T4, T3, tetraiodothyroacetic acid (tetrac), 3,3′,5′ T3 and 3,3′ T2.

The radioactive spots on the two-dimensional chromatograms were cut out and the radioactivity was measured in the scintillation counter. The percentage of total radioactivity on the chromatogram which was present in each spot was then calculated. In a similar manner, radioautograms were made of two-dimensional chromatograms of the radioactive T4 after it was simply diluted with 0.02 N NH_4OH and a sample of 5 μl or

less was pipetted on to the filter paper. The strips were developed for 20 to 24 hours in BDA. The radioactive areas were located by scanning with a chromatogram scanner (Forro) or by exposure of film (Eastman Linagraph Ortho film). Radioactive bands were eluted with 0.02N NH_4OH. I^{131} compounds were incuabted with β-glucuronidase in phosphate buffer at pH 6.5 at 37° C for 60 minutes. Four volumes of methanol-acetone (1:1) was added, and the mixture was centrifuged. The extract was dried in vacuo, and the I^{131} compounds were separated by paper chromatography.

I^{131} compounds also were incubated overnight with Mylase P in M/15 phosphate buffer at pH 6.0 at 50°, then extracted in the same way and separated by paper chromatography. I^{131} compounds that were hydrolyzed by β-glucuronidase were not altered by Mylase P, and those that were hydrolyzed by Mylase P were not altered by β-glucuronidase. I^{131} compounds after elution from the paper chromatograms were sometimes dissolved in N HCl and boiled for 20 minutes; then the hydrolytic products were separated on paper chromatograms.

Chromatograms of some specimens were made also on the kieselguhr column with 4:1 butanol-chloroform-0.5N NaOH, then with 9:1 butanol-propanol-0.5N NaOH, and finally with water as described elsewhere (11). Comparison of the effects of Mylase P on the radioactive peaks from the column chromatograms with the effects of this enzyme on the radioactive spots of the paper chromatograms has resolved a discrepancy noted earlier in a study of the urine of the dehepatized dog (11). At that time an appreciable portion of the early asymmetric peak of the column was thought to be T4, although little, if any, free T4 was found on paper chromatograms of the urine. In the present study, we have found that sulfoconjugates and possibly other unidentified conjugates come off the kieselguhr column early and merge with or overlap the T4 peak. These conjugates appear on the paper chromatograms to the right or lower right of iodide and are in large part hydrolyzable with Mylase P. When small samples of urine (1 ml. or less) are chromatographed, the glucurono-conjugates move off the column after the change in solvents from butanol-chloroform-0.5N NaOH to butanol-propanol-0.5N NaOH. When extracts of 6 ml. or 20 ml. of urine are chromatographed on the usual 6 gm. of kieselguhr the glucuronides may move off the column with the first solvent. A good separation of T4 glucuronide from 3,3′,5′ T3 glucuronide then has been obtained.

RESULTS

A brief summary of results is given in Table 1 to present the over-all picture. Details follow.

Biliary Fistula.—The average fraction of the dose of I^{131} excreted within 24 hours after the injection of I^{131}-labeled T4 into 10 dogs with biliary fistulas was 18.5% in the bile and 20.0% in the urine. The amount circulating in the plasma at the end of this period was 7.2% (Table 2). The five dogs with biliary fistulas given injections of T4 within 2 hours of the operation excreted 19.4 to 38.1% of the dose in the urine, whereas the five dogs given the T4 1 to 2 days after the operation excreted only 7.6 to 11.5% of the dose in the urine. Only one dog in each of these two groups was given Lugol's solution to inhibit uptake of iodide by the thyroid. None of the dehepatized dogs were given Lugol's solution, since the operation has been shown to decrease thyroidal uptake of iodide. No difference was noted in the output of I^{131} in the bile when the dogs with biliary fistulas were given T4 within 2 hours of the operation and on the following day. Dehepatized dogs excreted much less I^{131} in the urine than dogs with biliary fistulas

TABLE 1. CONJUGATED I^{131} COMPOUNDS AFTER INJECTION OF THYROXINE IN BILE FROM DOGS WITH BILIARY FISTULAS AND IN URINE OF DEHEPATIZED DOGS

I^{131} compounds	Percent* of total excreted in			
	Bile† from dogs with biliary fistulas		Urine‡ from dehepatized dogs	
Total	100		100	
G region	43		41	
T4 glucuronide		22		15
T3 glucuronide		3		
3,3′,5′ T3 glucuronide		10		20
Tetrac glucuronide		4		
M region	18		31	
T4 sulfate				2 (3§)
3,3′,5′ T3 sulfate		1		4 (14§)
3,3′ T2 sulfate		6		16 (19§)
Other M		6		10 (0§)

* Percentages have been smoothed in this table to show over-all picture.
† Derived from average of amounts of values excreted in bile during first 24 hours after dose (Tables 3 and 4).
‡ Derived from average amounts excreted in urine in 24 hours after dose (Table 5).
§ Percentages found when M region was hydrolyzed with N HCl instead of Mylase P.

which received T4 on the day of operation. The plasma levels in the dehepatized dogs were usually higher than those in dogs with biliary fistulas (Table 2).

When the I^{131} compounds excreted in bile were examined by two-dimensional paper chromatography, some free T4 was found. Smaller amounts of each of the triiodothyronines, T3 and 3,3′,5′ T3, were also found in the free state (Fig. 1, Table 3). Most of the I^{131} was found in conjugated form in radioactive spots to the right of and below the iodide. When the glucuronides (G) were incubated with β-glucuronidase, two major products were liberated, one of which coincided with the T4 marker, the other with the 3,3′,5′ T3 marker. Minor products were T3 and possibly also some tetrac. T4 glucuronide and 3,3′,5′ T3 glucuronide are difficult to separate completely on the paper chromatograms run in BDA. However, good separation was obtained when a concentrated extract of 6 ml. of urine had been chromatographed on a kieselguhr column. When T4 glucuronide from such a column was incubated with β-glucuronidase and the hydrolytic products were separated on a paper chromatogram, a radioactive T4

TABLE 2. DISTRIBUTION OF I^{131} IN DOGS IN THE 24 HOURS AFTER INJECTION OF T4

Fluid	Percent of dose of I^{131} in body fluids of:	
	10 dogs with biliary fistulas	6 dehepatized dogs
Plasma*	7.2 ± 0.6†	11.4 ± 1.9
Urine	20.0 ± 4.0	13.6 ± 1.9
Bile	18.5 ± 2.1	

* Plasma volume is calculated as 4.5% of body weight.
† Mean values and standard error of the mean.

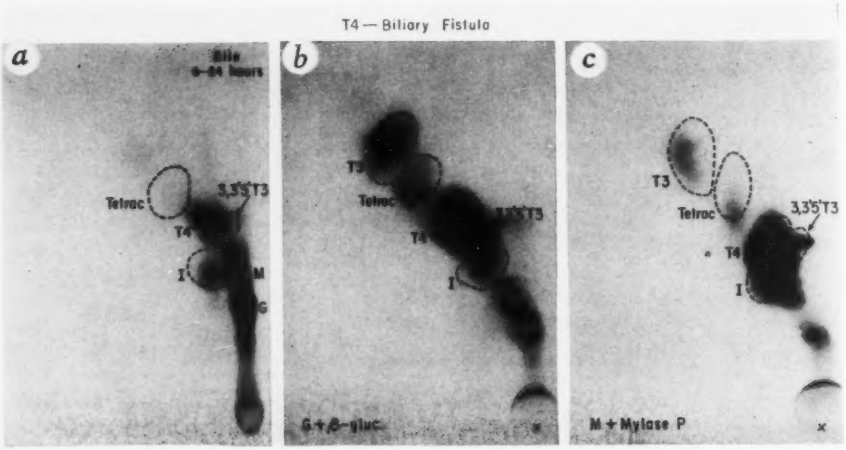

FIG. 1. Radioautograms of typical two-dimensional chromatograms of bile.

a. Of the I^{131} injected as T4, 13.4% was excreted in the bile in 6 to 24 hours. Of the amount excreted, 37.7% was present as glucuronoconjugates (G), 13% as sulfoconjugates (M), 3% as iodide, 4% as 3,3′,5′ T3, 18% as T4, and 17% as T3.

b. Following hydrolysis of the glucuronoconjugates with β-glucuronidase, 60% of the I^{131} in the hydrolysate traveled with the T4 marker, 14% with the 3,3′,5′ marker, 4% with the tetrac marker, and 8% with the T3.

c. Following hydrolysis of the sulfoconjugate with Mylase P, 46% of the I^{131} traveled with the T4 marker, 17% with the 3,3′,5′ T3 marker, and 4% with the T3 marker; 2% at the lower edge of the tetrac marker was 3,3′ T2.

spot appeared without the 3,3′,5′ T3 spot. Likewise, 3,3′,5′ T3 without T4 was liberated by the action of β-glucuronidase on 3,3′,5′ T3 glucuronide. An average of 42.6% of the I^{131} in the bile was found in the glucuronides; approximately a half of this I^{131} was in T4 glucuronide and a fourth in 3,3′,5′ T3 glucuronide (Tables 1 and 4).

An average of 17.9% of the I^{131} in the bile was found in the sulfate frac-

TABLE 3. UNCONJUGATED THYRONINES IN BILE

Dog	Hours after injection	Percent of biliary I^{131}		
		T3	T4	3,3′,5′ T3
1	0–6	4.4	12.5	3.8
	6–24	5.6	13.3	4.4
2	0–6		21.3	4.5
3	0–6		24.2	7.4
4	0–6		14.0	5.8
	6–24		18.2	5.0
5	0–6		4.0	6.9
	6–24		3.2	2.9

TABLE 4. I^{131} IN CONJUGATES OF BILE*

Percent of biliary I^{131}	Products liberated by β-glucuronidase, percent of total glucuronides			
	T3	T4	3,3′,5′ T3	Tetrac
In glucuronides 42.6 ± 2.3 (9)	7.6 ± 0.9	52.2 ± 4.1	23.0 ± 3.3	3.7 ± 0.3
In Mylase-hydrolyzable region	Products liberated by Mylase P, percent of M region			
	3,3′ T2		3,3′,5′ T3	Not hydrolyzed
17.9 ± 1.4 (9)	30.9 ± 3.8 (5)		4.2 ± 1.1 (5)	33.8 ± 7.4 (5)

* Mean values are given for the major conjugates of bile collected either during the first 6 hours or the next 18 hours after the injection of T4. Number after ± is standard error; number in parentheses is number of specimens.

tion (M). When this was incubated with Mylase P, the major hydrolytic product was found to be 3,3′ T2. A minor product was 3,3′,5′ T3. One third of the I^{131} remained in an unhydrolyzed conjugate. If, however, the sulfate fraction was boiled with N HCl, hydrolysis was complete and much more 3,3′,5′ T3 was liberated. It thus appears that 3,3′,5′ T3 is excreted in the bile conjugated with at least three different substances: glucuronic acid, sulfuric acid, and an unidentified substance. Only occasionally was a small spot of radioactivity found in the T4 region when the sulfate fraction of dog bile was examined (Fig. 1*c*).

Hepatectomy.—Dehepatized dogs made the same organic metabolites of T4 as the dogs with livers. In fact the radioautograms of the I^{131} compounds in urine of the dehepatized dogs were similar to those of bile (Fig. 2). Glucuronides of both T4 and 3,3′,5′ T3 were excreted in the urine; the relative amount of 3,3′,5′ T3 was greater in urine than in bile (Table 5). The percentage of the I^{131} found in the sulfoconjugate region was also higher in urine. When the region was hydrolyzed with Mylase P, the most abundant hydrolytic product was 3,3′ T2, but both T4 and 3,3′,5′ T3 were found. When the region was hydrolyzed with acid, much more 3,3′5′ T3 was liberated.

Much of the I^{131} in the plasma of dehepatized dogs 24 hours after injection of T4 was found to be unchanged T4 (Table 6). A significant amount of 3,3′,5′ T3 was also present in the free state with a similar amount of inorganic iodide. Small amounts of glucuronides were present, and these increased from 6 to 24 hours after the injection of T4. Larger increases in the sulfoconjugates were found.

When the commercial radioactive T4 was chromatographed directly, a number of spots of low radioactivity were always found in addition to the T4 spot of high radioactivity. The spot close to T4 in the 3,3′,5′ T3 position accounted for 4 to 11%; T3, for 1 to 5% and iodide, for 4 to 10% of the radioactivity. When the T4 spot was eluted and rechromatographed, a T4 spot of high radioactivity was found without a spot of radioactive

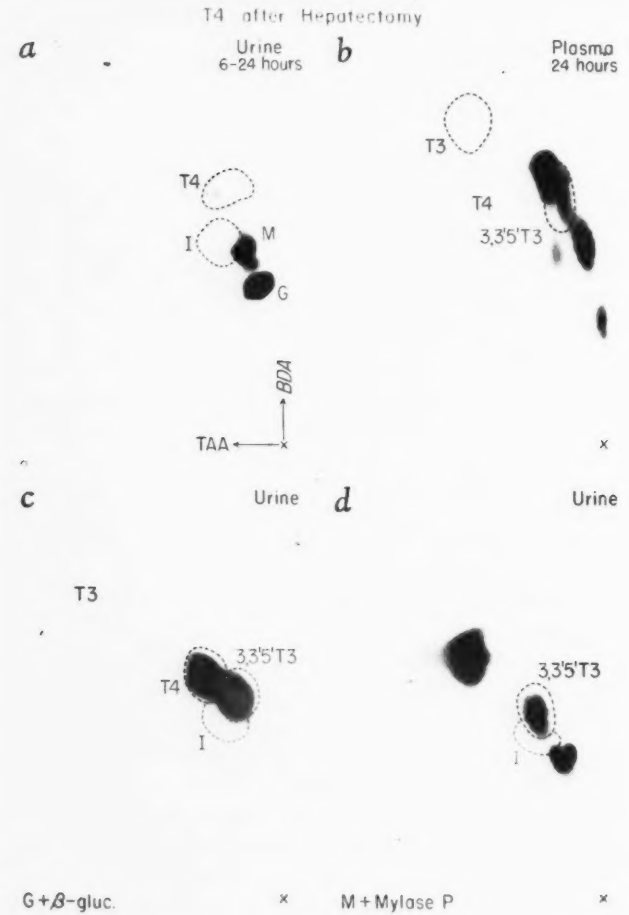

FIG. 2. Radioautograms of two-dimensional chromatograms of urine and plasma of a dehepatized dog.

a. I^{131} compounds excreted in urine from 6 to 24 hours after the injection of T4 (8.6% of dose). Of the I^{131}, 57% was found in spot G which included both T4 glucuronide and 3,3′,5′ T3 glucuronide; 24% was found in spot M which included a small amount of 3,3′,5′ T3 sulfate and a larger amount of 3,3′ T2 sulfate.

b. I^{131} compounds in plasma 24 hours after injection of T4 (6% of dose). Of the I^{131}, 41% was present as free T4, and 13% as free 3,3′,5′ T3, at the right of the T4.

c. Hydrolytic products liberated from urine by action of β-glucuronidase on spot G. Of the I^{131}, 58% was 3,3′,5′ T3, and 34% was T4. Although both markers T4 and 3,3′,5′ T3 are indicated by the dashed lines on the chromatograms, each was added to only one of duplicate chromatograms.

d. Hydrolytic products liberated by Mylase P from spot M from urine. Of the I^{131} 15% was 3,3′,5′ T3, 64% was 3,3′ T2 (unlabeled), and 19% at the lower edge of the I marker was unhydrolyzed.

TABLE 5. URINARY CONJUGATES IN DEHEPATIZED DOGS

Dog	Hours after injection	I^{131} in urine, percent of dose	Percent of urinary I^{131} in glucuronides	T3	T4	3,3′,5′ T3	3,3′ T2	Unhydrolyzed
				Products liberated by β-glucuronidase, percent of glucuronides				
6	0–6	2.7						
	6–24	11.1	43.9		38.0	62.0		
7	0–6	2.7			55.9	38.2		
	6–24	11.1	61.6		28.1	64.5		
8	0–24	6.8	49.8		42.2	34.3		
9	0–6	1.3	22.0		37.3	39.2		
	6–24	15.6	31.0					
10	0–6	3.1	29.9		43.4	28.8		
	6–24	16.9	39.2		38.2	45.2		
			In Mylase hydrolyzable region (M region)	Products liberated by Mylase P, percent of M region				
6	6–24	11.1	48.2					
7	6–24	11.1	27.4		14.4	18.4	57.5	18.8
8	0–24	6.8	23.1			14.8	47.7	17.7
9	0–6	1.3	48.1	2.7	8.6	7.3	43.2	25.4
	6–24	15.6	45.1					
10	0–6	3.1	44.4		1.6	7.7	41.4	37.6
	6–24	16.9	41.5	1.8	1.6	9.4	28.2	31.8
				Products liberated by N HCl, percent of M region				
9	0–24			4.1	3.8	32.1	54.2	
10	0–6			4.2	13.5	24.4	40.1	
	6–24			2.6	5.4	39.0	37.0	

3,3′,5′ T3, but did contain a small spot of radioactive iodide.

These experiments suggest that some breakdown of T4 to 3,3′,5′ T3 occurs from self-radiation; some breakdown of T4 to iodide occurs both from self-radiation and chromatography. The increasing amounts of 3,3′,5′ T3 found in the dog after injection of T4 appear to have a metabolic origin.

COMMENT

Partial deiodination of T4 occurs readily in the dog with or without a liver. On the basis of the amount of 3,3′,5′ T3 which accumulates in the bile of the dog with a biliary fistula or in the plasma and urine of the dehepatized dog, in free or conjugated form after the injection of T4, it appears that partial deiodination of T4 proceeds more readily from position 5 than 5′. Thus, much more of the T4 becomes inactivated by the forma-

tion of 3,3′,5′ T3, rather than activated by formation of T3. A great difference in the calorigenic and antigoitrogenic assays of these two triiodothyronines has been shown by Money and associates (9) and by Stasilli, Kroc, and Meltzer (13).

Recognition of 3,3′,5′ T3 glucuronide as the important metabolite of T4 (described earlier as an unidentified compound [10]) in bile, and also in plasma and urine of dehepatized dogs, became possible when 3,3′,5′ T3 became available for use as a marker. 3,3′,5′ T3 has been found in small amounts in the free state and in large amounts in the conjugated state. This suggests that partial deiodination of T4 may precede conjugation.

TABLE 6. I^{131} IN PLASMA OF DEHEPATIZED DOGS AFTER INJECTION OF T4

Dog	Hours after injection	I^{131} in plasma, percent of dose	I^{131} content, percent of plasma I^{131}				
			Glucuronide region	Mylase-hydrolyzable region	Free T4	Free 3,3′,5′ T3	Iodide
7	6	19.4	3.4	4.3	72.9	6.4	7.2
	24	7.8	6.0	25.9	38.9	15.4	6.8
8	6	37.1	2.2	6.6	59.7	8.2	17.7
	24	13.1	6.8	39.1	19.6	10.4	16.6
9	6	29.5	3.2	7.4	60.5	11.2	13.6
	24	11.0	6.3	30.4	34.0	17.8	8.2
10	6	21.0	4.7	6.2	39.2	5.6	37.9
	24	9.4	8.0	38.6	33.0		15.2

3,3′,5′ T3 like T4 is conjugated chiefly with glucuronic acid, but small amounts of 3,3′,5′ T3 are also conjugated with sulfate. Since larger amounts of 3,3′,5′ T3 are liberated from the sulfoconjugate region of paper chromatograms by acid hydrolysis than by hydrolysis with Mylase P, 3,3′,5′ T3 probably is also conjugated with a third, as yet unidentified, substance.

Recognition of 3,3′ T2 as an important metabolite of T4 in bile, and also in plasma and urine of the dehepatized dog, indicates that partial deiodination of T4 may be progressive. 3,3′ T2 is a common metabolite of both T4 and T3 (1). 3,3′ T2 was found as a sulfoconjugate which was hydrolyzable with Mylase P. Roche and co-workers (14) have reported the finding of minute amounts of 3,3′ T2 in the free state in kidney of rats after administration of both isomers of T3 (3,5′,3′ and 3,3′,5′).

Partial deiodination of T4 occurs readily in extrahepatic tissues as well as in the whole dog. Our study shows, moreover, that more 3,3′,5′ T3 glucuronide was found in the dehepatized dog than in the normal dog; this is due probably both to more prolonged contact with extrahepatic tissues and to lack of metabolism of the 3,3′,5′ T3 by the liver. Extrahepatic tissues remove some iodine from position 5′ of T4 as well as from position 5 and thus make 3,3′ T2. Less of this radioactive iodine is liber-

ted by the dehepatized dog than by the normal dog as shown by the much smaller amount of radioactive iodide excreted in the urine of the dehepatized dog.

It has become increasingly apparent that organic metabolites of both T4 and T3 are found chiefly in conjugated form in bile or after hepatectomy in urine and blood. The ability of the liver to concentrate the metabolites which are excreted in the bile provides a great advantage for the detection of these substances as does the accumulation of organic metabolites of T4 in blood and urine after liver removal because further degradation is limited. Our studies indicate that partial deiodination of the iodothyronines is of primary importance in the metabolism of these substances in the dog. Alterations in the side chain seem to be secondary and, therefore, of minor importance.

3,3′,5′ T3 has not been considered an important metabolite of T4 in *in vitro* studies, which have been concerned in general with the process of deiodination (15) and not with conjugation. Conversion of T4 to T3 has been demonstrated in slices of kidney from the rat (16, 17) and from man (18). Conversion of T4 to tetrac has been observed in kidney homogenates (19, 20). Small amounts of conjugates also have been found in kidney homogenates by Etling and Barker (20). Tata (21), who found that T3 was the chief metabolite of T4 in muscle, mentioned that deiodination was more complicated in the liver and kidney than in muscle, since several products were formed in the former. Deiodination of 3′,5′ labeled T4 (22, 23) with liberation of radioactive iodide occurs readily in liver extracts, homogenates or slices, but there is only slight deiodination of 3,5 labeled T4 (23). Yamazaki and Slingerland (24) and Lissitzky and co-workers (25) have shown that liver slices will remove all iodide atoms from T4 with the formation of thyronine.

Roche and associates (4) and Closon (5) have demonstrated that esterification of T4 in the rat proceeds chiefly with glucuronic acid and to a small degree with sulfuric acid. Roche and Michel (26) found small amounts of both T4 sulfate and T4 glucuronide in plasma and lymph from the thoracic duct of dogs after injection of T4. Our observation of small amounts of T3 sulfate in the bile of the dog after injection of T4 agrees with observations made on the rat by Roche, Michel and Gruson (27).

Iodine atoms in position 3′ and 5′ of thyronines are generally considered more labile than those in 3 and 5 positions (15). Only the 3′ and 5′ atoms of T4 are readily exchangeable with I^{131} (28). Positions 3′ and 5′ of thyronine are preferentially iodinated (29), but they are much more stable to catalytic hydrogenation with Raney nickel (30). In a small series of rats, Roche, Michel and Tata (31) found somewhat more radioactive iodide in the urine after injection of T4 labeled with I^{131} in positions 3′ and 5′ than after injection of T4 labeled in positions 3 and 5.

3,3′,5′ T3, administered to rats, is readily conjugated with glucuronic

acid, and the conjugate is excreted in the bile according to Roche and associates (32). Dunn and Stanbury (33) have found that 3,3′,5′ T3 is rapidly degraded when administered to man; 50% is excreted in the urine as iodide in 24 hours. Free 3,3′,5′ T3 has been shown to have 75% of the effect of T4 in suppressing thyroidal uptake of I^{131} (9, 13). Rawson and co-workers (34) reported that in large doses 3,3′,5′ T3 decreases the basal metabolic rate of patients with classic Graves' disease. Pittman and Barker (35) found that it inhibits the peripheral utilization of T4. The presence of appreciable amounts of 3,3′,5′ T3 conjugates in bile could reduce the effectiveness of the enterohepatic circulation (36) of iodine compounds of bile in the maintenance of the level of thyroxine in blood.

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STUDIES ON STEROIDAL ENOL ETHERS: AN ATTEMPT TO DISSOCIATE PROGESTATIONAL FROM CONTRACEPTIVE ACTIVITY IN ORAL GESTAGENS

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ABSTRACT

As a further step in a program of biological investigations of enol ethers of Δ^4-3-ketosteroid compounds, 17α-acetoxyprogesterone cyclopentyl enol ether was tested orally on rabbits for progestational effects and on rats for other endocrine activities. The compound proved very effective in the Clauberg assay and in maintaining pregnancy in spayed rabbits, but it was practically inactive in the intrauterine assay. On the other hand it failed to interfere with estrus, mating, ovulation, pregnancy and delivery at term in the rat. In addition, neither impairment of gonadotropic pituitary function, nor masculinizing action on the external genitalia of fetuses was observed. The bearing of these findings on the possibility of having developed a pure progestational substance is discussed.

THE unusual biological properties of the enol ethers of Δ^4-3-keto steroidal compounds have been described in several previous publications (1–5). Recent experiments with the cyclopentyl enol ether of 17α-acetoxyprogesterone have also proved interesting, its activity being apparently that of a purely progestational substance. This compound together with other progestins has been assayed for the following properties: a) ability to produce a secretory endometrium in rabbits, b) ability to maintain pregnancy in rabbits, c) contraceptive activity in rats, d) possible masculinizing effects on female fetuses in rats, and, e) pituitary gonadotropin inhibition in parabiotic rats.

MATERIALS

The steroids used in this experiment were the following: estradiol-17β, progesterone, 17α-acetoxyprogesterone (AP), 6α-methyl-17α-acetoxyprogesterone (MAP), 17α-acetoxyprogesterone cyclopentyl enol ether (APc-5),[1] and testosterone propionate (TP).

As it was shown in an earlier study (6) that an oily medium enhances oral activity of steroid compounds, sesame oil was chosen as the vehicle. The steroids were administered daily, at selected doses, either parenterally in 0.2 cc. sesame oil to both rabbits and rats

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[1] The physical constants of 17α-acetoxyprogesterone cyclopentyl enol ether are as follows: m.p. 158–159.5°; [α]D −142°. The present figures amend those reported in a previous paper (1).

or orally (via a stomach tube) in a volume of 1.0 cc. of sesame oil to rabbits and 0.2 cc. to rats.

METHODS AND RESULTS

Endometrial stimulation

The ability of the progestins to produce a secretory endometrium was tested by the Clauberg method. Immature female rabbits, weighing 700–900 gm., were first primed with 6 daily subcutaneous injections of 5 μg of estradiol. From the 7th day, the test compounds were administered for 5 days either subcutaneously or orally; during this time estrogen dosage was reduced to 0.5 μg daily. The animals were killed 24 hours after the last administration, and their uteri removed, sectioned, stained with haematoxylin-eosin, and graded from 1 to 4 according to the McPhail technique (7) for the progestational reaction. Table 1 presents the data obtained from

TABLE 1. AVERAGE ENDOMETRIAL RESPONSES TO 17α-ACETOXYPROGESTERONE CYCLOPENTYL ENOL ETHER (APc-5) IN THE RABBIT (CLAUBERG TEST) IN COMPARISON WITH PROGESTERONE AND 17α-ACETOXYPROGESTERONE (AP)

Compound	Route of administration	Daily dose/animal		McPhail index	No. of animals
		mg.	μmoles		
Progesterone	subcutaneous	0.031	0.1	0	5
		0.062	0.2	1.5	5
		0.125	0.4	2.7	5
		0.251	0.8	3.5	5
AP	oral	1.116	3	2.2	5
		1.860	5	3.2	5
APc-5	oral	0.220	0.5	2.8	11
		0.660	1.5	3.2	9

the administration of progesterone, 17α-acetoxyprogesterone (AP) and 17α-acetoxyprogesterone cyclopentyl enol ether (APc-5). As Table 1 shows, APc-5 orally administered exhibits an activity almost similar to that of progesterone given subcutaneously in equimolar doses, but it is, by far, more potent than AP.

The ability of test compounds to induce a progestational proliferation of the endometrium was also studied by means of intrauterine assay, according to the method of McGinty *et al.* (8). In this experiment the steroids were injected into a segment of immature rabbit uterus, previously primed with estrogens. The animals were sacrificed 3 days later, and the uterine segments were prepared for histological study and graded subsequently on the basis of endometrial gland development (7). It is evident from the data shown in Table 2 that APc-5, when administered locally, is less active than either progesterone or AP.

Maintenance of pregnancy

In order to investigate the pregnancy maintaining properties of test compounds, female rabbits were ovariectomized on the tenth day of pregnancy and treated daily with progestins for 7 days.

TABLE 2. ENDOMETRIAL RESPONSES TO 17α-ACETOXYPROGESTERONE CYCLOPENTYL ENOL ETHER (APc-5) IN THE RABBIT (McGINTY TEST) IN COMPARISON WITH PROGESTERONE AND 17α-ACETOXYPROGESTERONE (AP)

Compound	Route of administration	Dose/animal*		McPhail index
		μg	μmoles	
Progesterone	intrauterine	6.28	0.02	3
		62.8	0.2	3
		628	2	3
AP	intrauterine	7.44	0.02	0
		74.4	0.2	1
		744	2	2
APc-5	intrauterine	8.8	0.02	0
		88	0.2	0
		880	2	1

* 1 rabbit per group.

At autopsy both living and dead fetuses were counted. In Table 3 the number of living fetuses is expressed as a percentage of the total number of implants. In addition, Table 3 indicates the degree of resorption of the dead fetuses using the following notations: + refers to initial, ++ to advanced, and +++ to subtotal resorption.

Examination of the results showed that APc-5, given orally, was more effective than progesterone, given subcutaneously, as a pregnancy maintaining compound. MAP, administered orally, was almost as potent as APc-5; on the other hand, AP had little or no pregnancy sustaining properties at the doses used.

Contraceptive activity

The method used for the assay of the contraceptive activity of the progestins is similar to that described by Saunders and Drill (9). Virgin female albino rats, weighing about 150 gm., were observed for a week and daily

TABLE 3. EFFECTIVENESS OF 17α-ACETOXYPROGESTERONE CYCLOPENTYL ENOL ETHER (APc-5) IN MAINTAINING PREGNANCY IN SPAYED RABBITS IN COMPARISON WITH PROGESTERONE, 17α-ACETOXYPROGESTERONE (AP) AND 6α-METHYL-17α ACETOXYPROGESTERONE (MAP)

Compound	Route of administration	Dose/kg./day		Living fetuses (as % of total implants)*	Degree of resorption of dead fetuses
		mg.	μmoles		
Vehicle	oral	—	—	0	+++
Progesterone	subcutaneous	0.628	2	50	++
		1.256	4	67	+
		2.512	8	70	+
AP	oral	1.860	5	0	+++
		3.720	10	8	++
MAP	oral	0.386	1	35	+
		0.772	2	53	++
APc-5	oral	0.440	1	37	+
		0.880	2	75	+
		1.320	3	90	+

* Average responses of 4 animals per group.

aginal smears were made in order to ascertain the presence of normal strus cycle.

Treatment with progestins was then started and after a second week the female rats ere caged with males, known to be fertile, at a ratio of 5 females to 2 males. Smears, ken daily, were examined for cornified epithelial cells and/or spermatozoa until pregncy was firmly established. The males were withdrawn on the 15th day of cohabitaon. Then the females were caged singly and checked periodically for their litters. The eatment with progestins was terminated 21 days after the last day of cohabitation, nless, of course, delivery occurred before that time.

In this study, APc-5 was compared with progesterone and testosterone propionate (TP), both given subcutaneously, and with AP and MAP, both given orally (Table 4).

At the doses used, neither progesterone, nor AP, nor APc-5 revealed any inhibitory effects on estrus, mating, fertilization, or delivery at term; on the other hand, MAP administration produced different effects. Only at the lower dosage did MAP allow some females to have estrus and mating, but none of these treated females bore litters. A similar pattern of effects was shown by TP injected rats; in this case the only difference was that some of the animals mated, although smears did not show estrous transformations in any case.

Since progesterone was known to be contraceptive in rats at certain dose levels, it seemed possible that APc-5 could also have this effect if given at higher doses. Thus, a second set of experiments employing 4 and 8 μmoles of APc-5 per animal and 4 μmoles of progesterone was undertaken. From the results (presented in Table 5) it is seen that although progesterone prevented more than half of the treated animals from reproducing, APc-5 even at the higher doses did not interfere with the normal reproductive capacity of the female rats. In this experiment only fertility records were kept.

Masculinizing effects

The possible masculinizing action of APc-5 was studied by observing the newborn rats from the contraceptive activity experiment. Since this type of examination was undertaken somewhat late in the course of the previous experiment, only a part of the litters appearing in Tables 4 and 5 could be utilized for the study of masculinizing effects. Virilization was judged by measuring ano-genital distances. Newborns with ano-genital distances under 2 mm. were considered to be females and those with a distance greater than 3.5 mm. regarded as males (Table 6). When the distance was between 2 and 3.5 mm., the young were classified provisionally as intersexuals; however, laparotomy showed that they consistently had ovaries. The newborn rats from APc-5 treated mothers were compared with young born from progesterone and MAP-treated mothers. As in the contraceptivity experiment no female treated with MAP became preg-

TABLE 4. EFFECT OF 17α-ACETOXYPROGESTERONE CYCLOPENTYL ENOL ETHER (AP c-5) UPON THE ESTROUS CYCLE AND REPRODUCTIVE BEHAVIOUR OF FEMALE RATS IN COMPARISON WITH PROGESTERONE, 17α-ACETOXYPROGESTERONE (AP), 6α-METHYL-17α-ACETOXYPROGESTERONE (MAP) AND TESTOSTERONE PROPIONATE (TP)

Compound	Route of administration	Daily dose/animal		No. of animals			Days between cohabitation and mating	Days between mating and delivery	No. of litters*	No. of young		Average weight of young (gm.)
		mg.	μmoles	Treated	With estrus inhibition	With mating inhibition				Total	Per litter	
Vehicle	oral	—	—	10	0	0	2.6±0.3†	21.5±0.1†	8	70	8.7±0.8†	5.8
Progesterone	subcutaneous	0.157	0.5	10	0	0	3.3±0.7	22.4±0.1	10	82	8.2±1.1	5.8
AP	oral	0.372	1	10	0	0	4.3±0.9	21.5±0.1	7	65	9.3±0.9	5.9
		0.744	2	10	0	0	3.6±1.0	21.4±0.3	8	74	9.2±0.5	5.7
APc-5	oral	0.440	1	10	0	0	6.5±1.3	21.7±0.2	7	69	9.8±0.5	5.6
		0.880	2	10	0	0	3.4±0.8	21.9±0.3	8	80	10.0±0.8	5.8
MAP	oral	0.193	0.5	10	6	8	4.5±3.5	—	0	0	0	—
		0.386	1	10	10	10	∞	—	0	0	0	—
TP	subcutaneous	0.344	1	10	10	5	6.0±1.4	—	0	0	0	—

* All obtained from spontaneous delivery.
† Mean ± Standard Error.

TABLE 5. EFFECTS OF PROGESTERONE AND 17α-ACETOXYPROGESTERONE CYCLOPENTYL ENOL ETHER (APe-5) UPON THE REPRODUCTIVE BEHAVIOUR OF FEMALE RATS

Compound	Route of administration	Daily dose/animal		No. of treated females	No. of litters*	No. of young	
		mg.	μmoles			Total	Per litter
Vehicle	oral	—	—	10	9	99	11.0 ± 0.9†
Progesterone	subcutaneous	1.256	4	10	4	44	11.0 ± 1.1
APe-5	oral	1.760	4	10	7	69	9.8 ± 0.8
		3.520	8	10	8	76	9.5 ± 1.2

* All obtained from spontaneous delivery.
† Mean ± Standard Error.

nant, it was necessary to start treatment with this steroid in already pregnant rats. The method described by Bur *et al.* (10) was used with some modifications as follows: Virgin female albino rats were mated, and the first day in which spermatozoa appeared in the vaginal smear was regarded as day 1 of pregnancy. MAP was given from the 12th day to the 22nd day of pregnancy. No spontaneous parturition occurred in the MAP-treated group, and therefore the young had to be removed by means of a caesarian section. The resulting data, presented in Table 6, show that, unlike MAP, progesterone and APe-5 did not cause detectable masculinization of young female rats.

Pituitary gonadotropin inhibition

The effect of APe-5 upon pituitary gonadotrophic activity was studied in parabiotic immature rats. Male and female rats, weighing 65 to 70 gm., were placed in parabiosis according to Bunster and Meyer (11). At the time of union, the male partner was castrated.

Progesterone, MAP, and APe-5 were given to the male parabiont once daily for 10 days, beginning with the day of the operation. The animals were sacrificed on the 11th day; the uteri and ovaries were weighed to the nearest 0.5 mg. (Table 7). Using these weights as a criterion, neither progesterone given subcutaneously nor APe-5 given orally was able to inhibit pituitary gonadotropin secretion at the doses used. MAP, on the other hand, effectively maintained low both the uterine and ovarian weights at doses equimolar to APe-5.

DISCUSSION

The described experiments show that APe-5 is a potent oral progestational agent, because of its ability to produce a secretory endometrium by means of the Clauberg test and to maintain pregnancy in ovariectomized rabbits. According to this investigation, its potency is similar to that of progesterone given subcutaneously and is far greater than that of AP given orally. This latter finding is not unexpected due to the well-known oral potentiation exhibited by Δ^4-3-ketosteroidal enol ethers in comparison to their parent compounds (1–5).

TABLE 6. EFFECT OF 17α-ACETOXYPROGESTERONE CYCLOPENTYL ENOL ETHER (APc-5), 6α-METHYL-17α-ACETOXYPROGESTERONE (MAP) AND PROGESTERONE, GIVEN DURING FETAL LIFE, ON THE ANO-GENITAL DISTANCE OF 1-DAY-OLD RATS

Compound	Route of administration	No. of treated mothers	Daily dose/animal		Duration of treatment (days)	Litters						Delivery*
						Females		Intersexuals		Males		
			mg.	μmoles		No. of young	Ano-genital distance (mm.)	No. of young	Ano-genital distance (mm.)	No. of young	Ano-genital distance (mm.)	
Vehicle	oral	7	—	—	~30†	26	1.44 ± 0.06‡	0	—	28	3.94 ± 0.11‡	S
Progesterone	subcutaneous	2	1.256	4	~30†	6	1.53 ± 0.13	0	—	7	4.14 ± 0.24	S
MAP	oral	3	1.158	3	10§	0	—	12	2.2 ± 0.1‡	12	4.7 ± 0.1	C
APc-5	oral	7	0.880	2	~30†	27	1.46 ± 0.05	0	—	32	4.28 ± 0.12	S
		7	1.760	4	~30†	23	1.65 ± 0.08	0	—	33	4.06 ± 0.09	S
		5	3.520	8	~30†	23	1.49 ± 0.26	0	—	19	4.06 ± 0.13	S

* S spontaneous, C by Caesarean section.
† From 7th day before cohabitation until delivery.
‡ Mean ± Standard Error.
§ 12th–22d day of pregnancy.

TABLE 7. EFFECTIVENESS OF 17α-ACETOXYPROGESTERONE CYCLOPENTYL ENOL ETHER (APc-5), 6α-METHYL-17α-ACETOXYPROGESTERONE (MAP) AND PROGESTERONE IN BLOCKING GONADOTROPHIC ACTIVITY IN THE PARABIOTIC RAT*

Compound	Route of administration	Daily dose/animal		Body weight of pairs in gm.		Average organ weights in mg.	
		mg.	μmoles	Initial	Final	Uterus	Ovaries
Vehicle (intact males)	oral	—	—	116	159	49.1± 5.5†	38.0± 1.2†
Vehicle (castrated males)	oral	—	—	118	158	141.3±11.5	126.5±12.0
Progesterone	subcutaneous	0.314	1	123	169	140.4±46.0	192.8±17.0
MAP	oral	0.386	1	118	154	98.6±16.7	93.2±21.0
		1.930	5	120	149	83.8±13.0	67.6±11.0
APc-5	oral	0.440	1	103	145	133.1±12.5	137.8±22.0
		2.200	5	117	157	133.2±12.5	177.0±10.8

* 10 pairs of male and female albino rats per group.
† Mean ± Standard Error.

Furthermore, APc-5 is almost ineffective in the intrauterine assay; this finding, however, is not surprising either, since it is also known that enol ethers are nearly inactive when given parenterally (1–3). Thus, the failure of APc-5 to elicit a response when injected directly into the lumen of the uterus provides further evidence that steroidal enol ethers are scarcely active *per se*, but are probably transformed into more active compounds following oral administration.

According to Saunders and Elton (12) and Drill (13), only those compounds which are active in the McGinty test (intrauterine assay) should be able to maintain pregnancy in ovariectomized animals. APc-5 is, in this respect, apparently an exception.

In sharp contrast to its high progestational activity in the Clauberg test and its maintenance of pregnancy, APc-5 seems to be lacking other endocrine effects, possessed by most oral progestational compounds. We refer mainly to the findings obtained in the fertility study. Actually, this experiment was a composite one, and the following effects on: estrus, ovulation, fertilization, course and duration of pregnancy, parturition, litter size, and fetal weight are taken into consideration. None of these parameters were adversely affected by treatment with APc-5; it is known, however, that other orally active progestins, as well as progesterone itself, interfere with some stages of the female reproductive cycle. Pincus and coworkers have extensively studied progestational compounds for antifertility properties and the reader is referred to their publications (14–21) for detailed information.

Among the orally active compounds AP has been shown to be a weak ovulation inhibitor (22), while it is effective when administered parenterally (23). Given orally, AP has been reported to be only 0.03 times as potent as 17α-ethynil-19-nortestosterone in rabbits (22). Furthermore, relatively high doses of AP were required to inhibit estrus (not necessarily ovulation) in bitches (24) and gilts (25). Moreover, in the present study, AP revealed itself to be devoid of a clear-cut action on the reproductive behavior of female rats, at least at the dosage levels employed. Whether

the poor activity of AP in inhibiting ovulation may be just a reflection of its apparently low progestational activity, cannot be stated at present, since there is a remarkable discrepancy in the literature as to its relative potency as an oral progestogen (see 22 and 26 in contrast to 27). Nevertheless, allowing for species differences, it would appear fairly clear from the present experiments that formation of the cyclopentyl enol ether, though enhancing the oral activity of AP as a progestational agent, does not increase its anti-ovulatory properties. It may be noted here that 17α-hydroxy-progesterone caproate also appears to be incapable of interfering with estrous cycle, mating, ovulation and litter size in the rat (28).

A relevant point for discussion is the failure of APc-5 to inhibit pituitary gonadotropin secretion. This raises the question as to whether the absence of antiovulatory effects is dependent upon the lack of pituitary inhibiting action by that steroid. Since 17α-hydroxyprogesterone caproate, which is inactive as an antiovulatory agent, is also devoid of significant pituitary inhibition, as judged by the ovarian weight response of parabiont female rats (29), it would seem plausible to assume a causal relationship between these two properties, as far as both APc-5 and 17α-hydroxyprogesterone caproate are concerned. Such a direct relationship, however, cannot apply to progesterone, which is an effective ovulation inhibitor, but inactive in the parabiosis test (29).

It is of interest, finally, to note the complete absence of masculinization of female fetuses even from large doses of APc-5. This is in contrast to the evident androgenic action exerted by MAP, as shown by the present experiments which are in agreement with the findings of Revesz *et al.* (30).

When all the biological properties of APc-5 described in these experiments are brought together for consideration, this compound then seems to be a unique oral progestational agent. It produces, in the uterine mucosa, anatomical modifications which are necessary for the nidation of the fertilized ovum, and, in addition, it is capable of supporting pregnancy in ovariectomized animals; therefore, APc-5 is, without a doubt, a true progestin. On the other hand, it appears to be devoid of other endocrine influences, such as: inhibiting action on estrus, mating, ovulation, nidation, delivery at term, and pituitary gonadotropin secretion, and so may be regarded purely as a progestational agent.

If APc-5 is to be thought of merely as a progestational agent, then the philosophical concept of a gestagen—a compound whose progestational properties *sensu strictiori* are bound to its contraceptive activities—would have to be revised. There are numerous well-known steroids which are effective ovulation inhibitors with little or no progestational activity. The studies on APc-5, as well as on 17α-hydroxyprogesterone caproate, suggest that the opposite may also be true, that is that there are compounds which are progestational agents without being contraceptive. Possible explana-

tions and further correlations of these observed phenomena will be the object of future study.

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EFFECTS OF FAT-MOBILIZING EXTRACT FROM HUMAN URINE ON METABOLISM OF GLUCOSE IN RAT ADIPOSE TISSUE[1]

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ABSTRACT

Fat-mobilizing substance (FMS), a small peptide isolated from urine of carbohydrate deprived humans, alters carbohydrate metabolism in isolated epididymal adipose tissue in a pattern similar to that of epinephrine and preparations of ACTH and growth hormone. It is inferred that FMS primarily mobilizes fatty acids from adipose tissue by directly augmenting the lipolytic reaction.

IN 1947, Weil and Stetten (1) reported an increase in liver lipid content in mice receiving extracts of urine obtained from fasted rabbits. Chalmers, Kekwick, Pawan and Smith (2) have isolated a similar fraction from urine from humans with intact pituitaries who were either fasted or fed a low carbohydrate diet. A recent report (3) has further characterized this human material. It is a small peptide with a molecular weight of less than 18,000, and after acid hydrolysis yields histidine, phenylalanine, leucine, serine, cystine, aspartic acid and a trace of alanine. Biological studies in mice have shown, following subcutaneous injection, an increase in liver fat and a prompt fall in blood sugar followed by an increase in blood ketones. Prolonged treatment results in a decrease in carcass fat. *In vitro* the material accelerates free fatty acid release from incubated adipose tissue in concentrations as low as 0.1 to 1.0 μg/ml. (3). This material has appropriately been named "fat-mobilizing substance" (FMS).

Recent studies on adipose tissue have shown a characteristic metabolic pattern shared by several hormones (ACTH, epinephrine, growth hormone) which accelerate the *in vitro* release of free fatty acids from adipose tissue (4, 5, 6). This pattern includes: 1) a primary increase in the rate of release of free fatty acids into the medium (if an acceptor such as albumin is present), 2) an increase in glucose uptake and an increase in the incorporation of glucose carbon into glyceride-glycerol, 3) a relative decrease in

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TABLE 1. EFFECTS OF FMS ON THE METABOLISM OF GLUCOSE-U-C^{14} IN RAT ADIPOSE TISSUE *in vitro*. FMS 10 μg/ml., GLUCOSE 5mM

Values in μM glucose/mg. N/3 hours. Means of 8 experiments with standard errors.

	CO_2	Glyceride-glycerol	Fatty acids	Glycogen
FMS	1.90 ± 0.21	0.96 ± 0.09	0.25 ± 0.06	0.004 ± 0.001
Control	1.17 ± 0.17	0.42 ± 0.06	0.29 ± 0.11	0.008 ± 0.002

the metabolism of glucose over the direct oxidative pathway, and 4) an increase in the oxidation of glucose by the tricarboxylic acid cycle as calculated from the metabolism of specifically labeled substrates (4, 5). In addition, in the presence of insulin, there is an even greater increase in glucose incorporation into glyceride-glycerol and the insulin effect on increasing glycogen synthesis is inhibited. The studies to be reported in this paper demonstrate that this same sequence of metabolic events occurs in adipose tissue incubated in the presence of fat-mobilizing substance (FMS).

EXPERIMENTAL

Adipose tissue fragments weighing from 100 to 200 mg. were excised from epididymal fat pads of normal fed rats (Wistar strain from Albino Farms), each animal serving as its own control for each of the experimental variables. Techniques of incubation, isolation of products and determination of specific activity have all been described (7, 8, 9). FMS was isolated from human urine according to previously described techniques (3) and added to the Krebs-Ringer bicarbonate buffer just prior to incubation. Labeled glucose was purchased from New England Nuclear Corporation, and crystalline insulin was obtained from Eli Lilly and Co. through the courtesy of Dr. W. R. Kirtley.

RESULTS

In Table 1 are listed results from adipose tissue incubated in the presence of uniformly-labeled glucose with and without FMS, 10 μg/ml. In the presence of FMS there is an increase in oxidation of glucose to CO_2 and incorporation of glucose carbon into glyceride-glycerol. There is no significant effect on glucose carbon incorporation into fatty acids or glycogen. Table 2 lists the effects of FMS on the metabolism of glucose-1-C^{14} and glucose-6-C^{14}. In control tissues, the usual preponderance of oxidation of carbon-1 to CO_2, compared to that of carbon-6, is noted. Likewise, there is the usual ratio of carbon-1 to carbon-6 of 0.5 in fatty acids, suggesting

TABLE 2. EFFECTS OF FMS ON THE METABOLISM OF GLUCOSE-1-C^{14} AND GLUCOSE-6-C^{14} IN RAT ADIPOSE TISSUE *in vitro*

FMS 10 μg/ml., glucose 5mM. Values in μM glucose/mg. N/3 hours. Means of 7 experiments with standard errors.

	CO_2	Glyceride-glycerol	Fatty acids	Glycogen
FMS				
Glucose-1-C^{14}	2.38 ± 0.52	2.64 ± 0.65	0.16 ± 0.05	0.013 ± 0.002
Glucose-6-C^{14}	2.30 ± 0.48	2.16 ± 0.74	0.13 ± 0.06	0.007 ± 0.003
Control				
Glucose-1-C^{14}	1.94 ± 0.31	0.74 ± 0.09	0.26 ± 0.09	0.018 ± 0.008
Glucose-6-C^{14}	0.58 ± 0.07	0.87 ± 0.06	0.51 ± 0.18	0.022 ± 0.006

TABLE 3. EFFECTS OF FMS WITH AND WITHOUT INSULIN ON THE METABOLISM OF GLUCOSE-U-C^{14} IN RAT ADIPOSE TISSUE *in vitro*

FMS 10 μg/ml., insulin 0.1 units/ml., glucose 5mM. Values in μM glucose/mg. N/3 hours. Means of 4 experiments with standard errors.

	CO_2	Glyceride-glycerol	Fatty acids	Glycogen
FMS+Insulin	5.99±0.49	5.41±0.33	2.41±0.42	0.008±0.002
FMS	1.50±0.26	0.93±0.17	0.35±0.07	0.002±0.001
Insulin	4.49±0.51	1.08±0.12	2.55±0.48	0.108±0.019
Control	0.98±0.33	0.54±0.08	0.38±0.20	0.005±0.001

that half the glucose is metabolized via a pathway with loss of carbon-1 relative to carbon-6. In the presence of FMS, carbon-6 oxidation to CO_2 is increased markedly and is equal to that of carbon-1. There is also a marked increase in glucose incorporation into glyceride-glycerol from both glucose carbons. Recoveries of carbon-1 and carbon-6 in fatty acids are now approximately equal suggesting lack of operation of any specific metabolic pathway with loss of one end of the glucose molecule compared to the other.

In Table 3 are listed results of experiments with and without insulin and FMS. The expected marked increase in the oxidation of glucose-U-C^{14} with insulin is noted as well as the marked increase in incorporation into glycogen and fatty acids. FMS in the presence of insulin increases even more the oxidation of glucose to CO_2 and induces a marked increase in the incorporation of glucose carbon into glyceride-glycerol. The insulin effect on glycogen is completely inhibited by FMS, whereas that on fatty acids is apparently unaffected. Similar findings but with specifically-labeled glucose are summarized in Table 4, demonstrating again that FMS tends to inhibit any preferential metabolism of one end of the glucose molecule compared to the other, even in the presence of an insulin-induced increase in glucose uptake. Since the effects of FMS are most marked in the presence of insulin, adipose tissue fragments were incubated in the presence of insulin and varying concentrations of FMS. These experiments are summarized in Figure 1 and show significant metabolic effects at 1 μg/ml.

TABLE 4. EFFECTS OF FMS WITH AND WITHOUT INSULIN ON THE METABOLISM OF GLUCOSE-1-C^{14} AND GLUCOSE-6-C^{14} IN RAT ADIPOSE TISSUE *in vitro*

FMS 10 μg/ml., insulin 0.1 unit/ml., glucose 5mM. Values in μM glucose/mg. N/3 hours. Means of 3 experiments with standard errors.

	CO_2	Glyceride-glycerol	Fatty acids	Glycogen
FMS+Insulin				
Glucose-1-C^{14}	4.13±1.70	4.82±0.44	0.371±0.312	0.020±0.006
Glucose-6-C^{14}	4.45±0.87	4.88±0.55	0.451±0.152	0.019±0.004
FMS				
Glucose-1-C^{14}	1.23±0.31	0.78±0.23	0.152±0.135	0.012±0.005
Glucose-6-C^{14}	0.85±0.19	0.74±0.16	0.141±0.072	0.002±0.001
Insulin				
Glucose-1-C^{14}	4.57±1.24	0.86±0.12	1.092±0.520	0.195±0.065
Glucose-6-C^{14}	0.63±0.10	1.13±0.10	1.792±0.61	0.175±0.053
Control				
Glucose-1-C^{14}	1.18±0.04	0.63±0.07	0.034±0.014	0.009±0.001
Glucose-6-C^{14}	0.40±0.02	0.61±0.65	0.049±0.015	0.013±0.009

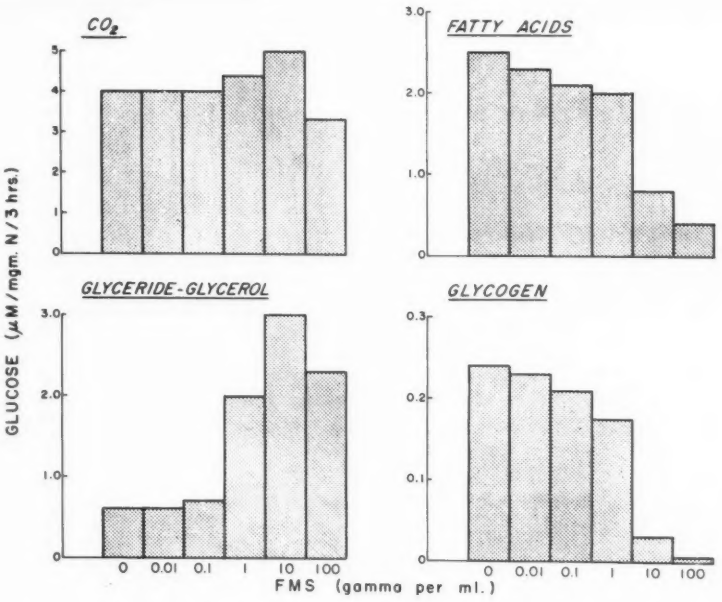

FIG. 1. Metabolism of glucose-U-C^{14} in rat adipose tissue *in vitro* in the presence of insulin and varying concentrations of FMS. Insulin 0.1 unit/ml.

DISCUSSION

The physiological effects of FMS on glucose metabolism in isolated adipose tissue are therefore similar to those previously reported for epinephrine and preparations of ACTH and growth hormone (4, 5, 6). The interpretation at present is that these hormones primarily accelerate lipolysis and do not exert their action via inhibition of esterification (4, 5, 6). The rise in intracellular fatty acids resulting from accelerated lipolysis induces increased glucose uptake and glyceride-glycerol synthesis and increased metabolism of glucose via the Embden-Meyerhof (glycolytic) pathway and oxidation by the tricarboxylic acid pathway (4, 5). There is usually an inhibition of glucose incorporation into tissue fatty acids in the presence of these hormones, but in occasional experiments no inhibition is observed.

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NOTES AND COMMENTS

AN ENDOCRINE MECHANISM INVOLVED IN TESTICULAR CHANGES PRODUCED BY A DL-ETHIONINE SUPPLEMENTED DIET: THE EFFECT ON EXTRATESTICULAR SOURCES OF 17-KS EXCRETION[1,2]

ABSTRACT

The effect of *dl*-ethionine on extratesticular androgenic sources in castrated rats was studied by means of the total daily urinary excretion of 17-ketosteroids.

In castrated rats, the continual ingestion of ethionine, despite its cumulative effect, had no affect on the rate of excretion of urinary 17-KS during the 24 days of administration. With respect to the total daily excretion of 17-KS, ethionine did not appear to affect any extratesticular androgenic sources (adrenal).

Castrated non-treated rats gaining up to 80% of their initial weight in 24 days, had the same rate of excretion of 17-KS throughout the experimental period. Despite a continuous loss of weight in castrated ethionine-treated rats, the daily urinary excretion of 17-KS was not affected. Thus a change in weight does not affect the 17-KS excretion in non-treated or ethionine-treated castrated animals.

Mild changes in the liver histology do not affect liver capacity to metabolize 17-KS in castrated ethionine-treated male rats.

It has been demonstrated previously that *dl*-ethionine has a damaging effect on the seminiferous epithelium of the rat (1–5). It has been shown that *dl*-ethionine reduces the excretion of urinary 17-ketosteroids (17-KS) by $\frac{2}{3}$ (5). The purpose of the present study was to examine the effect of *dl*-ethionine on extratesticular sources of 17-KS precursors.

MATERIAL AND METHODS

Albino male rats (Sprague-Dawley) weighing about 120 gm. at the beginning of the experiment were used. The rats were fed *ad libitum* on a diet of Purina lab chow. Three groups of rats each comprising six animals were used. Two groups were subjected to bilateral orchiectomy and were allowed to recuperate for two weeks.

The animals were treated as follows:

Group A.—Intact, untreated controls.

Group K.—Castrated and given aqueous solution of 0.5% ethionine[3] in drinking water *ad libitum*.

Group L.—Castrated and given water *ad libitum*.

It was estimated that each rat in Group K ingested about 70 to 100 mg. of ethionine daily. Urine was pooled daily from each of the groups for evaluation of 17-KS according to the Holtorff-Koch modification of the Zimmerman reaction (6).

The animals were sacrificed on the 24th day. Liver, pancreas, and adrenal tissue removed at that time were fixed in formalin and submitted for histological processing and morphological evaluation.

RESULTS

Castrated rats ingesting ethionine in the drinking water showed fluctuations in the excretion of urinary 17-KS, but the rate of excretion was quite uniform throughout the experimental period although at $\frac{1}{3}$ the normal control rate. No difference in the rate of 17-KS excretion was noted in the first 12 days or during the second 12 days of the ex-

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[2] This study was presented in part at the 35th Anniversary Congress Pan American Medical Association, Mexico City, May 2–11, 1960.

[3] Nutritional Biochemicals Corporation, Cleveland, Ohio.

TABLE 1. MEAN VALUE μG 17/KS/24 HR./RAT

Group	Days	
	1–12	13–24
A—Control	58	53
K—Castrated + Ethionine	22	22
L—Castrated	19	21

periment (Table 1). This was despite the fact that the animals had a mean loss, from initial weight, of 34% on the 12th day, and 26% on the 24th day (Table 2).

Histologically, the liver showed only a mild proliferation of oval cells (7) around the portal spaces, although the pancreas was severely atrophic. The adrenals were decreased in size and depleted of fats. The normal histologic pattern and relationship of the different zones were preserved.

In castrated rats not given ethionine the rate of excretion of urinary 17-KS was reduced by ⅔ as compared to the rate of 17-KS excretion in intact control animals (Group A, Table 1). In all groups the rate of 17-KS excretion was the same in the first 12 days as during the second 12 days of the experiment. Despite a weight gain of 40% by the 12th day of the experiment, which increased to 80% by the 24th day, the rate of 17-KS excretion was not affected.

No histological changes were encountered in the liver, pancreas or adrenal.

DISCUSSION

In a previous study (5) ethionine was found to decrease the rate of 17-KS excretion before any morphological changes could be observed in the testis. The question presented itself as to what extent this decline in 17-KS could be attributed to an ethionine effect on androgens originating from the adrenal (8). Since bilateral orchiectomy in the albino male rat decreased the total daily excretion of 17-KS by about two-thirds one may conclude that about two-thirds of the urinary 17-KS originate from the testes. The fact that the administration of *dl*-ethionine to castrated albino male rats (Gr.K.), did not change the total daily urinary 17-KS excretion from the total daily excretion in castrated non-treated rats it is also apparent that this amino acid does not affect 17-KS originating from extratesticular sources, namely the adrenals.

Despite the fact that on the 24th day the animals in Group K had a mean loss of 40% of their initial weight and the animals of Group L had a mean gain of 80% of their initial weight the mean excretion values for 17-KS were nearly identical. One may conclude therefore that in castrated albino male rats the nutritional state, as reflected by the change in weight, does not affect the total excretion of 17-KS.

In trying to prove that the androgens originating from the testis are produced by the interstitial glandular tissue, certain authors (8) have thought it convincing if atrophy of the seminiferous epithelium was induced without damage to interstitial tissue and androgenic material continued to be secreted by the modified testis. This evidence ap-

TABLE 2. MEAN WEIGHT AND PERCENT OF CHANGE IN WEIGHT

Group and type	Weight in gm.			Percent of change in weight	
	Initial	Orchiectomy (12th day)	Sacrifice (24th day)	(12th day)	(24th day)
A—Control	130	190	222	+46%	+71%
K—Castrated + Ethionine	133	88	99	−34%	−26%
L—Castrated	127	178	229	+40%	+80%

pears insufficient in light of the fact that androgens are elaborated also by the adrenal (8). On the other hand our previous study (5) has demonstrated that the morphological appearance of the interstitial tissue is not always a reliable index of its functional capacity. Thus when one employs x-rays (9) radium (10) or artificial cryptorchidism (8) in order to produce atrophy of the seminiferous epithelium the associated normal appearing Leydig cells are not necessarily functioning normally and an intact adrenal may conceivably mask a functional Leydig cell deficiency.

In as much as the present study suggests that the 17-KS from adrenal origin are not decreased in the ethionine-treated castrated male rats it would appear that the Leydig cells are the only source of androgens in this species. This is in agreement with our previous study (5) in which it was shown that the rate of excretion of 17-KS also declined by about $\frac{2}{3}$ in ethionine-treated intact rats.

Further corroboration will be necessary in order to study the possible enzymatic mechanism by which *dl*-ethionine affects the testicular androgens and not the adrenal androgens despite the fact that both organs are, supposedly, secreting related androgenic substances.

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EFFECT OF DL-THYROXINE ON THE PBI AND BMR OF RATS POISONED WITH AMINONITRILES[1,2]

ABSTRACT

Alterations in the weight of the thyroid glands, levels of serum protein-bound iodine (PBI) and basal metabolic rates (BMR) were observed in rats poisoned with methyleneaminoacetonitrile (MAAN) or fed a 50% *Lathyrus odoratus* diet. Thyroxine prevents the connective tissue abnormalities (bone lesions or exostoses) induced with MAAN but has no effect on *L. odoratus* diet. It also prevents the weight increase of the thyroids produced either by MAAN or *L. odoratus*. Rats poisoned

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[1] Partly reported at the 4th International Congress of Biochemistry in Vienna, Austria, Sept. 1958. Pergamon Press, Inc., New York, N. Y. Sec. 8, No. 86, p. 100.

[2] The data presented in this paper are from the doctoral thesis of Sabit Gabay, University of Madrid, 1958.

both with MAAN or *L. odoratus* seeds exhibit an increase in the BMR. When thyroxine is administered either to normal or *L. odoratus* fed rats (which show no protection) the BMR and PBI increase significantly. Whereas thyroxine protects against MAAN poisoning PBI does not rise but BMR increases. An inhibition mechanism of the toxic effect of MAAN by thyroxine is suggested.

The increase in size of the thyroid glands repeatedly observed in rats fed *Lathyrus odoratus* (sweet-pea) seeds (1) induced us to study the effect of thyroidectomy, as well as the administration of DL-thyroxine (T4) in experimental lathyrism (odoratism) produced by methyleneaminoacetonitrile (MAAN).[3] It was exceedingly remarkable to see the aggravation when MAAN was given to thyroidectomized animals and the active protection when T4 was parenterally injected (2). In experiments where aminoacetonitrile (AAN)[2] was utilized (3) the same striking effects were observed. According to recent findings, the protection (4) is 50 to 100-fold stronger in action when triiodothyronine is used. On the other hand it was reported that only a slight effect is observed when triiodothyronine is administered together with β-Aminopropionitrile (BAPN)[2] (5). It can be concluded that evidence so far reported does not explain materially the mechanism of action of thyroxine as a protecting agent. In the studies presented, an attempt is made to find an explanation for the action of this compound.

MATERIAL AND METHODS

In this investigation the effect of T4 on rats fed a 50% *L. odoratus* diet, and on animals treated orally with 5 mg./100 gm. body weight of MAAN was studied. Basal metabolic rates (BMR) and serum protein-bound iodine (PBI) estimations were made. To determine if the effect of T4 might be due to its iodine content, potassium iodide (KI) was used in order to see whether this compound could have a preventive effect on aminonitrile poisoning.

The experiment was carried out on 70 rats of the Wistar strain, 29–32 days of age, ranging initially from 43–58 gm. in weight and equally distributed as to sex. Animals were subsequently divided in 7 groups containing 10 rats each. The first 5 groups received a diet of 50% wheat meal,[4] the last two 50% *L. odoratus*.[5] Group 1 represents the normal. Group 2 consists of normals receiving daily 75 μg./100 gm. body weight of T4 subcutaneously. Groups 3, 4 and 5, received MAAN in the dose of 5 mg./100 gm. body weight/day, administered orally. In addition, KI was given to Group 5, in the dose of 6.25 μg./100 gm. body weight daily. Groups 4 and 7 were simultaneously receiving T4 in the same dose and by the same route as Group 2. Diets were given *ad libitum* and the rats were weighed every other day. Animals were housed in individual cages and daily diet intake was recorded. The duration of this experiment was 30–35 days.

The estimations of BMR were carried out, in duplicate (two consecutive days), on a representative group of 5 animals from each lot measuring the oxygen consumption by placing the animals into a vacuum desiccator connected with a small spirometer of the Krogh type. A Braun kymograph was used as recording device. At the end of the experiment animals were anesthetized with ether and blood was withdrawn through the hepatic vein. The blood of the rats in groups of two was pooled. Serum PBI determinations, in duplicate samples, were made according to the method of Connor *et al.* (6). Inorganic iodine was also estimated in the filtrates. The whole skin of every animal was excised and extended on a graph paper for use in calculating the BMR according to the animal's body surface. The thyroid glands were weighed on a precision torsion balance (Roller-Smith) immediately after they were extirpated.

RESULTS

As shown in Table 1 and Figure 1, T4 affords a total protection in rats poisoned with MAAN whereas no protection is seen in the *L. odoratus* fed group. MAAN, in addition to severe bone lesions, produces a hypertrophy of the thyroids. This increase in size of

[3] Methyleneaminoacetonitrile (MAAN): $CH_2{=}N{-}CH_2{-}CN$ synthesized according to "Organic Syntheses" (R. Adams and W. D. Lanhley, eds.) Coll. Vol. I, p. 347. John Wiley and Sons, New York, 1949. Aminoacetonitrile (AAN): $CH_2{-}NH_2{-}CN$; β-aminopropionitrile (BAPN): $NH_2{-}CH_2{-}CH_2{-}CN$.

[4] Constituted as follows: wheat meal 50%, casein 10%, starch 25%, sucrose 5%, salt mixture 4%, olive oil 2%, cod liver oil 2%, dried yeast 2%.

[5] Same as in footnote 3, except that 50% *L. odoratus* seed was substituted for 50% wheat meal.

TABLE 1. EFFECT OF DL-THYROXINE ON RATS POISONED WITH MAAN OR FED A DIET OF 50% *L. odoratus*

No.	Group	No. of rats	Food intake, gm./rat /day	Initial weight, gm.	Final weight, gm.	Thyroids (wet wgt), mg./100 gm.B.W.	Severity of bone lesions†
1	Normal	10	—	—	110	9.8±1.0*	0
2	DL-thyroxine (T4)	10	11.4	44	124	8.4±1.5	0
3	MAAN	10	6.7	43	82	14.4±3.6	5+
4	MAAN+T4	10	8.7	44	95	9.0±1.9	0
5	MAAN+KI	10	8.1	45	109	10.4±2.7	4+
6	Odoratus 50%	10	9.7	58	121	15.6±2.0	3+
7	Odoratus 50%+T4	10	12.0	52	113	7.1±1.8	3+

* Standard deviation.
† Ref: Figure 1.

the glands is also seen in the group receiving 50% *L. odoratus* diet, which confirms the previous observation (1). Thyroxine *per se* only produces a slight decrease of the weight of the thyroids ($P < 0.05 > 0.02$). This hormone, however, inhibits the increase in size produced both by MAAN and *L. odoratus*. KI does not protect against aminonitrile poisoning, thus demonstrating that the protective effect of T4 is not due to its iodine content.

The results of PBI and BMR determinations are shown in Table 2. PBI values in Group 1 are abnormally high for supposedly normal rats. This could be attributed to a high iodine content of the wheat meal diet. This is supported by the finding of rather high values of serum inorganic iodine in all groups. Both MAAN (with or without KI added) and thé *L. odoratus* diet increase the BMR approximately 50% (Groups 3, 5 and 6). Serum PBI however, shows a slight tendency to be lower than normal. (1–3 and 1–5 P, not significant; 1–6 $P < 0.01$). T4 *per se* (Group 2) produces a highly significant increase of both BMR and PBI, (1–2 $P < 0.001$). The PBI remains also elevated in Group 7 (50% *L. odoratus*+T4), (1–7 $P < 0.01 > 0.001$). When MAAN and T4 are given simultaneously (Group 4) the PBI values do not differ from those of normals. (1–4 P, not significant). BMR is increased in the same proportion in all groups receiving T4 (Groups 2, 4 and 7).

DISCUSSION

The increase in size of the thyroid glands produced by MAAN or *L. odoratus* diet is accompanied by an increase of peripheral oxygen consumption, whereas the serum PBI

TABLE 2. SERUM PBI AND BMR OF RATS IN EXPERIMENTAL ODORATISM

No.	Group	Number of pooled sera	Serum PBI,† μg/100 ml.	Inorganic μg/100 ml. iodine,	BMR, cal./m.²/hr.	Percent increase of BMR vs. normal
1	Normal	7	8.6±1.0*	4.0±0.8*	49	—
2	DL-thyroxine (T4)	4	19.4±1.5	7.7±1.7	120	145
3	MAAN	4	7.3±1.4	4.9±0.6	75	53
4	MAAN+T4	5	9.2±1.1	5.8±0.8	102	110
5	MAAN+KI	4	9.2±1.0	29.7±9.5	70	43
6	Odoratus 50%	3	6.8±0.5	4.8±1.0	77	58
7	Odoratus 50%+T4	3	12.8±1.7	6.4±0.5	113	113

* Standard deviation.
† Average of duplicate samples.
Statistical significance of PBI values: 1–2, $P < 0.001$. 1–3, 1–4 and 1–5, P not significant. 1–6, $P < 0.01$. 1–7, $P < 0.01$. 2–3 and 2–4, $P < 0.001$. 2–7, $P < 0.01$. 6–7, $P < 0.001$. 3–4, P not significant.

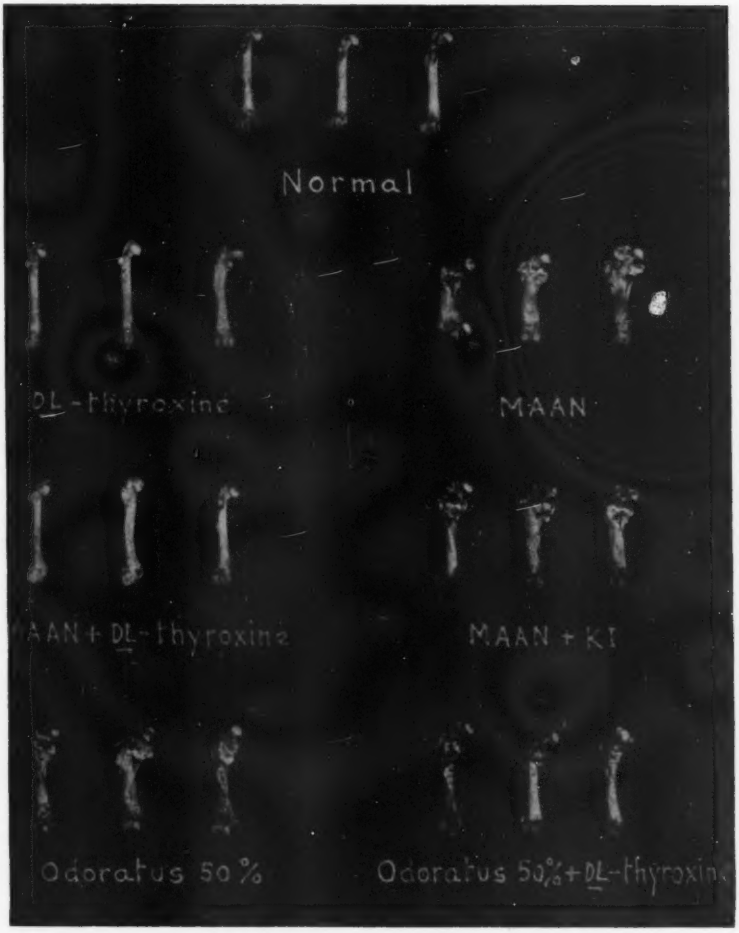

FIG. 1. Femurs of rats excised at the time that PBI determinations were carried out. Reference may be made to our previous report (7) relative to quantitative changes in connective tissue components and minerals content.

remains unchanged or tends to decrease. When T4 is given alone to normal rats a great increase of both BMR and serum PBI was observed. However, when T4 and MAAN are administered together, a total protection of the bone lesions is obtained, BMR increases but serum PBI does not rise. In the 7th group, 50% *L. odoratus* + T4, where protection does not exist, BMR and PBI remain elevated.

It seems therefore that the protective effect of T4 is in some way related to the unchanged PBI level. These data suggest that either a defect in the binding of serum thyroxine or an inhibition of the toxic effect of MAAN by T4 could explain this fact. This inhibition, if such is the case, does not seem to be related to the increased oxygen consumption induced by T4 since the BMR increases in the same proportion in the protected and non-protected animals. It seems likely that T4 counteracts the metabolic disturbances produced by MAAN (7).

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HISTOCHEMICAL STUDY OF OXIDATIVE ENZYMES IN THE HUMAN PARATHYROID[1]

ABSTRACT

The distribution of ten oxidative enzymes was studied in a parathyroid adenoma and in seven surgical biopsies of human parathyroid gland, using histochemical techniques. An intense enzymatic activity particularly of cytochrome oxidase, DPN and TPN diaphorase and of succinic dehydrogenase was found in the oxyphil cells contrasting with the weaker reaction observed in the chief cells. These results give support to the hypothesis that the oxyphil cells are not degenerated as commonly believed but metabolically active.

The human parathyroid gland in the adult is composed of two main types of epithelial cells: the chief cell and the oxyphil cell. It is commonly believed that the chief cell is the active secreting element of the gland while the oxyphil cell represents an involuted and degenerated form of chief cell (1). However Trier (2), using the electron

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microscope, has studied monkey parathyroid glands which resemble human parathyroids histologically and has shown that the oxyphil cells contain numerous mitochondria. In addition Tremblay and Pearse (3) have demonstrated, also in the monkey, by means of histochemical techniques, that the activity of several oxidative enzymes (DPN diaphorase, TPN diaphorase, succinic dehydrogenase, β-hydroxybutyric dehydrogenase and glucose-6-phosphate dehydrogenase) is strong in the oxyphil cells and weak in the chief cells. The present report is concerned with the histochemical distribution of ten oxidative enzymes in human parathyroid glands and in one parathyroid adenoma.

MATERIAL AND METHODS

The parathyroid adenoma was obtained surgically from a 53-year old woman with hyperparathyroidism. The tumor involved the lower left parathyroid gland and measured 1.8 cm in its greatest diameter. Histologically it was composed of enlarged chief cells among which were scattered isolated and small groups of oxyphil cells. In addition, seven small surgical biopsies of parathyroid gland were examined. Two of these specimens were obtained from the upper right and lower right parathyroid gland in the case of hyperparathyroidism. The other biopsies were taken from parathyroid glands identified in five patients undergoing thyroidectomy for nodular goiter or hyperplastic goiter. All the glands contained the two types of cells.

The specimens were frozen on metal chucks with dry ice immediately after surgical excision. Frozen sections 8 μ thick were cut in a cryostat. Diphosphopyridine nucleotide diaphorase (DPND), triphosphopyridine nucleotide diaphorase (TPND), succinic dehydrogenase, glucose-6-phosphate dehydrogenase, DPN- and TPN- dependant isocitric dehydrogenase, glutamic dehydrogenase, malic dehydrogenase and lactic dehydrogenase were determined using 2,2′-di-(p-nitrophenyl)-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′-biphenylene) ditetrazolium chloride (Nitro BT) or 3,5-diphenyl-2-(4,5-dimethylthiazol-2-yl) tetrazolium bromide (MTT) as final electron acceptor (4, 5). For the study of cytochrome oxidase the method of Burstone (6) was employed with α-naphthol and N-phenyl-p-phenylenediamine as reagents. After the determination of cytochrome oxidase and of the dehydrogenases with MTT, the identification of the oxyphil cells was accomplished as previously described, by destaining the sections with alcohol and restaining them with hematoxylin and eosin (3). This method could not be used with Nitro BT which gives, upon enzymatic reduction, a colored formazan insoluble in organic solvent. In this instance the oxyphil cells were identified by staining an adjacent section with hematoxylin and eosin.

RESULTS

The reaction for cytochrome oxidase (Figs. 1 and 3), succinic dehydrogenase (Fig. 5), DPN diaphorase (Fig. 6) and TPN diaphorase (see legend of Fig. 6), revealed an intense enzymatic activity in the oxyphil cells contrasting with a weaker activity in the chief cells. This strong reaction was found in the oxyphil cells contained in the adenoma (Figs. 1, 3, 5) as well as in the non-neoplastic glands (Fig. 6). The other dehydrogenases studied showed less activity in both types of cells; nevertheless the reactions were significantly stronger in the oxyphil cells than in the principal cells.

COMMENTS

There can be no doubt about the secretory capacity of the principal cells since they are the only cells present in some adenomas with hyperparathyroidism (7), in primary hyperplasia (1) and in the gland of some animals (8). The assumption that the oxyphil cells are involuted is based mainly on the observation that their number increases with age (1), and that adenomas composed entirely of these cells do not give rise to symptoms of hyperparathyroidism (7). However Tremblay and Pearse (3) have pointed out that it is difficult to reconcile this viewpoint with their finding of a high content of oxidative enzymes in the oxyphil cells of the monkey parathyroid. They thus suggested the possibility that the oxyphil cells are active and may be concerned with the elaboration of a secretory product not yet identified.

The present observation shows that the oxyphil cells in the human parathyroid gland are also rich in oxidative enzymes and thus give further support to the hypothesis that these cells are not degenerated but metabolically active.

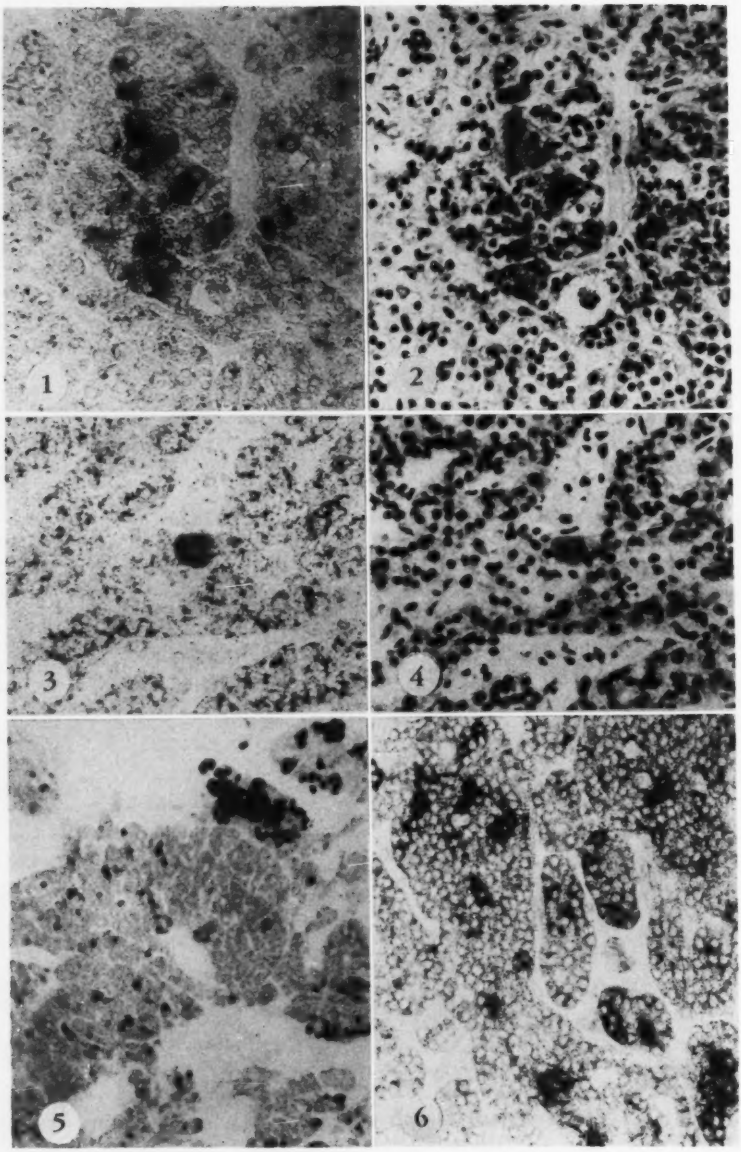

FIG. 1. Cytochrome oxidase reaction in the parathyroid adenoma. A group of oxyphil cells shows an intense activity contrasting with the weak reaction in the principal cells, ×210.

FIG. 2. The same section stained with hematoxylin and eosin. The strongly reactive cells are typical oxyphil cells with abundant granular cytoplasm, ×210.

FIG. 3. Parathyroid adenoma. Single oxyphil cell showing an intense cytochrome

Acknowledgment

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GROWTH HORMONE EFFECT ON THE BLOOD PLASMA PROTEINS IN THE PARAKEET[1,2]

ABSTRACT

Parakeets with pituitary adenomas or carcinomas show a characteristic marked increase in an albumin component of plasma protein. To determine if growth hormone injection results in this same characteristic increase, preparations of purified bovine, sheep, pig, monkey and human growth hormone were administered intramuscularly into the breasts of 3- to 6-month-old parakeets. The birds were bled before and on the third day after hormone injection. and relative protein components were measured by paper strip electrophoresis. Regardless of the source of growth hormone (bovine, sheep, pig, monkey or human) a highly significant "tumor-characteristic" increase was found. As in the case of tumor-bearing parakeets, the outstanding histologic finding in growth hormone injected birds was an accumulation of fat diffusely distributed throughout the liver lobules.

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[1] This experimental study was planned by Dr. Hans G. Schlumberger. During the course of the investigation Dr. Schlumberger was critically injured in an automobile accident and was unable to continue this research problem.

[2] Supported by a grant from the National Institutes of Health (C-3434).

oxidase activity among the weakly reactive chief cells, ×210.

FIG. 4. The same section stained with hematoxylin and eosin. The highly active cell is a typical oxyphil cell, ×210.

FIG. 5. Succinic dehydrogenase activity in the parathyroid adenoma. Isolated and clusters of oxyphil cells react intensely. The more numerous chief cells are only moderately active, ×40.

FIG. 6. DPN-diaphorase activity in a human parathyroid gland. Scattered oxyphil cells are strongly reactive. The chief cells show weaker activity. Similar results were observed with TPN diaphorase reaction. The nuclei contain no enzyme and appear as clear round spaces, ×210.

One of the most common tumors in the parakeet, *Melopsittacus undulatus*, is a neoplasm of the pituitary (1). Two of these tumors have been carried as serial transplants in this laboratory; one is now in the 29th passage (2) and the other in its 6th passage. All birds with the primary or transplanted pituitary tumors show a marked increase in one plasma protein component, a characteristic that has not been observed in normal birds or in parakeets with other types of tumors (3). Fox (4) identified this protein component by solubility criteria as an albumin.

The presence of a growth hormone secreted by the pituitary tumor is indicated by an increase in body and organ weights of tumor-bearing nestling parakeets and by the positive result of the hypophysectomized rat tibia test with an extract of the tumor (5).

This paper is a report of experiments designed to determine if intramuscular injection of growth hormones into normal parakeets results in a pituitary tumor- characteristic increase in an albumin component of blood plasma protein.

MATERIALS AND METHODS

Both male and female parakeets aged 3–6 months were anesthetized with an aqueous solution of 1.25 mg. (0.1 cc.) phenobarbital sodium injected intramuscularly into the breast. Anesthetized birds were bled via wing vein; the blood was collected in heparinized capillary tubes and was centrifuged in an International capillary micro-centrifuge, Model M.B. Blood plasma samples (0.006 ml./sample) were delivered by lambda pipettes onto a Spinco stripper and applied to the paper strips. The Spinco Model R paper electrophoresis system consisting of a Model R series D paper electrophoresis cell, Model R duostat and the Model R B Analytrol, was used throughout. Using Spinco veronal buffer at pH 8.6 as the electrolyte medium a constant current of 3 milliamperes per cell was applied to the paper for a period of 16 hours. After heat fixation at 120° C the proteins were stained with bromophenol blue and stained strips were scanned with the Analytrol. Protein components were separated as to peaks, integration units were counted and per cent of each component was calculated. Only those birds with initially normal protein albumin patterns (component 1 representing at least 8% and component 2 representing not more than 33% of the total protein) were used for these experiments.

Preliminary tests indicated that a single injection of 0.5 mg. purified bovine growth hormone was sufficient to bring about a marked increase in protein component 2 (albumin) by the 3rd day. In another preliminary experiment to determine when a significant increase in this albumin component first appears and how long this increase persists, 56 parakeets were bled via wing vein for normal protein patterns. Two weeks later 0.5 mg. of purified bovine growth hormone was injected intramuscularly and 8 birds were bled and then sacrificed on the 1st, 2nd, 3rd, 4th, 5th, 7th and 9th days post-injection. Statistical evaluation of protein components before and after hormone administration indicated that a significant increase was first observed on the 3rd day after hormone injection but was not found on the 4th, 5th, 7th or 9th day.

In separate experiments, preparations of purified bovine, sheep, pig, monkey and human growth hormones[3] were injected intramuscularly into the breasts of normal parakeets. The purified hormones were suspended in 0.9% sodium chloride so that the amount of inoculum per bird contained 0.5 mg. hormone. On the 3rd day after hormone injections the birds were bled and protein determinations were made as before. After wing vein bleeding the birds were killed by dislocating the cervical vertebrae; tissues were fixed in formalin and were sectioned for histological examination.

RESULTS

Mean per cent values of the four protein components in 97 parakeets 3 days after treatment with specific hormones are presented in Table 1. There was a statistically significant increase ($P < 0.001$) in protein component 2 with a corresponding decrease in the other components in all treated birds.

The significant histologic finding was a diffuse fatty metamorphosis in the liver of treated parakeets

DISCUSSION

A significant increase in the plasma protein component 2 seen in growth hormone injected parakeets on the 3rd day postinjection coincides with the first observable in-

[3] Courtesy of the Endocrinology Study Section, National Institutes of Health.

TABLE 1. MEAN PER CENT VALUES OF PLASMA PROTEINS IN BIRDS 3 DAYS AFTER INJECTION WITH PURIFIED GROWTH HORMONES

Hormones	No. of birds	Plasma protein components*			
		1	2	3	4
Bovine					
Before	50	21	26	23.5	22
After	50	16	41	15	18
P Value		<0.001	<0.001	<0.001	<0.001
Sheep					
Before	12	22.5	25	17	26
After	12	18	34	13	18.5
P Value		n.s.**	<0.001	<0.025	<0.05
Pig					
Before	12	19	27.5	17.5	30.5
After	12	19	35	14	17
P Value		n.s.	<0.001	<0.05	<0.005
Monkey					
Before	11	23.5	22	18.5	17.5
After	11	15.5	38	14	14
P Value		<0.001	<0.001	n.s.	n.s.
Human					
Before	12	18	26	22.5	29
After	12	13	43.5	12.5	17.5
P Value		n.s.	<0.001	<0.001	<0.01

* A fifth protein component was observed in 40 birds before and in 67 birds after hormone injection. Mean value in the former was 19% and in the latter was 18%, not a significant difference.

** n.s. = not significant.

crease in this component following pituitary tumor transplantation (3). Also like the plasma protein of parakeets bearing transplanted pituitary tumors, there was a relative decrease in the other three protein components without an obvious absolute decrease (3) (6). Using the techniques of immune electrophoresis and specific absorption of anti-sera Fox (4) showed that this protein component 2 is albumin rather than a unique protein present in the plasma of pituitary tumor-bearing parakeets. The diffuse distribution of fat throughout the liver lobules of treated birds was also similar to that seen in the liver cells of transplanted pituitary tumor-bearing parakeets (5).

The presence of a significant plasma albumin increase and a fatty metamorphosis in the liver cells of parakeets injected with purified growth hormone extract suggest the possibility that plasma paper strip electrophoresis might be used as another means of detecting the presence of growth hormone. Experiments designed to test this hypothesis are in progress.

Although a fifth plasma protein component was observed in 40 of the 97 birds prior to hormone injection and in 67 birds after hormone administration, there was no statistically significant relative increase in this component. The occasional appearance of a fifth plasma protein component in young parakeets (2–5 months old) prior to and following pituitary tumor transplantation has been observed in this laboratory previously (5). This random appearance of a fifth protein component in young parakeets is not clearly understood at the present time and awaits further study.

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STUDIES ON THE ASCORBIC ACID CONTENT OF THE ADRENAL OF THE BAT (*MYOTIS LUCIFUGUS*)[1]

ABSTRACT

The ascorbic acid content of the adrenals of the bat was determined in hibernating and active animals following epinephrine and ACTH treatment of captive animals. Non-captive bats were studied during hibernation and active states, and following arousal from hibernation. Captivity *per se* did not influence the adrenal vitamin content of hibernating or active bats. Among the captive animals, ACTH was effective in hibernating and epinephrine in active animals in decreasing the adrenal ascorbic acid. The most marked depletion of the adrenal vitamin occurred in the non-captive hibernating bats stressed by arousal, a finding well correlated with the blood corticosterone content reported for the hamster, another hibernating species, after similar treatment. The studies indicate a relationship of ascorbic acid to adrenal physiology of the bat comparable to that observed for some other species.

The recent, renewed interests in comparative endocrinology emphasize the need to study the humoral relationships in lower vertebrates and invertebrates to better understand the actions of hormones in all species. The endocrine physiology of native hibernating mammals has received little attention, obviously due to the poor adaptability of these animals to laboratory conditions.

The studies on the adrenal ascorbic acid of a number of species by Elton, *et al.* (1) revealed that this vitamin could be depleted from the glands of only some species by cold stress or adrenocorticotrophic hormone (ACTH). However, no special consideration was given to those species which normally hibernate during the winter months. It would be of interest to note the response of animals to stress or ACTH during both the hibernating and active states, and whether the ascorbic acid concentration of the adrenal of the bat reflects the physiological changes demonstrated for other species. With such objectives in mind, the following experiments were designed to study the effects of epinephrine and ACTH on the adrenal ascorbic acid of bats when either active or cold-induced hibernating during captivity. Other studies were initiated to determine adrenal activity of non-captive active and hibernating populations, and the effect of arousal from hibernation.

METHODS

All animals utilized in these experiments were adult females. The bats were captured during their diurnal roosting period from colonies occupying local caves or houses. The captive bats were maintained according to the procedure outlined by Krutzsch and Sulkin (2). Active animals were kept at a temperature of 30° C and a relative humidity of 68%. Artificial hibernation of captive bats was induced by placing them in a cold room at 5° C. Non-captive animals represent those bats which were sacrificed by decapitation immediately upon capture in the field. In every case, adrenal glands were removed, freed of extraneous connective tissue, and weighed on a torsion balance. Ascorbic acid concentration was determined by the method of Roe and Kuether (3). Captive bats were stressed by an injection of 100 μg of epinephrine or treated with 0.01 I.U. of ACTH (Armour) intraperitoneally per animal. Non-captive bats were stressed by bringing them from hibernation to an active state.

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[1] This investigation was supported by U.S.P.H.S. grants #RG 6599 and #A-1228(C_4).

RESULTS

The adrenal ascorbic acid concentration of the bat is not influenced by captivity. The active animals under both captive and non-captive conditions demonstrate comparable glandular vitamin contents (Groups IV and VIII, Table 1). Likewise, hibernating animals studied in a laboratory cold room or in the field show adrenal ascorbic acid values not significantly different (Groups I and VII).

When captured hibernating bats are treated with epinephrine, no changes in the adrenal vitamin content are noted (Groups I and II). However, the administration of ACTH to a hibernating bat results in a depletion of ascorbic acid of about 43% (Groups I and III, $P < .01$).

Captured bats maintained in an active state have higher adrenal ascorbic acid contents than similar hibernating animals (Groups I and IV, $P < .05$). In the case of these active animals, however, the epinephrine treatment appears to be more effective in depleting adrenal ascorbic acid (Groups IV and V, $P < .02$) than did the ACTH (Groups IV and VI, $P < .25$).

Among the non-captive groups studied, the active animals demonstrate a higher adrenal vitamin content than hibernating bats (Groups VII and VIII, $P < .005$), a finding similar to that mentioned above for captive groups. The most marked depletion of adrenal ascorbic acid occurs in the non-captive bats stressed by arousing them from hibernation to an active state. The vitamin content decreased about 82% (Groups VII and IX, $P < .001$).

DISCUSSION

The problem of whether or not ascorbic acid is related to the physiological activity of the adrenal cortex in the various species studied has been reviewed by Elton *et al.* (1). An examination of the adrenal vitamin values obtained in this study following endocrine treatment reveal a relationship of ascorbic acid in adrenal physiology of the bat comparable to that seen in rats and guinea pigs, species upon which most studies have been performed.

Due to the seasonal selection of the animals, all of the captive female bats were pregnant whereas those females in the non-captive groups were not. Nevertheless, active or hibernating animals under either condition revealed similar adrenal ascorbic acid concentrations. Pregnancy in the captive bats was not reflected in any adrenal vitamin alteration.

It is notable that the ACTH was effective in causing a vitamin depletion in the captive hibernating bats, but no changes were found following epinephrine treatment. Perhaps in hibernating animals the lowered metabolic activity reduces pituitary production of ACTH so that adrenal stimulation is observed only after exogenous trophic hormone administration. Among the captive active bats, significant depletions of ascorbic acid were noted after epinephrine rather than ACTH treatment. A possible explanation might be that the epinephrine stimulated endogenous ACTH production which was more effective in reducing the adrenal vitamin content than the exogenous trophic hormone, a substance readily metabolized in the active animal. One might also theorize that in the active bat the administered ACTH facilitated the rate of ascorbic acid repletion in the adrenal to balance the vitamin depletion, a situation comparable to that described by Kessler and Leathem (4) for lack of adrenal cholesterol depletion in mice following ACTH treatment.

The marked depletion of adrenal ascorbic acid in the non-captive bats stressed by arousing from hibernation coincides well with data available on blood corticosteroids of similarly stressed hibernating hamsters. Denyes and Horwood (5) reported that the extremely low blood corticosterone level in the hibernating hamster is rapidly increased upon arousal. If the ascorbic acid in the adrenal of the bat is intimately associated with

TABLE 1. ASCORBIC ACID CONCENTRATION OF BAT ADRENALS UNDER VARIOUS PHYSIOLOGICAL STATES

Condition	Groups	Adr. asc. acid (μg/100 mg. tissue)	P[4]
Captive[1]	I Hibernating (13)	139 ± 15*	
	II Hibernating + epinephrine (13)	147 ± 22	
	III Hibernating + ACTH (12)	79 ± 14	< .01
	IV Active (25)	190 ± 16	< .05†
	V Active + epinephrine (22)	140 ± 12	< .02
	VI Active + ACTH (20)	163 ± 14	< .25
Non-Captive[2]	VII Hibernating (9)	122 ± 9	
	VIII Active (10)	208 ± 23	< .005†
	IX Stressed[3] (10)	22 ± 13	< .001

* Standard error.
[1] Adult pregnant female bats obtained during summer months.
[2] Adult normal females obtained during winter months.
[3] Stressed by bringing from hibernation to active state.
( ) Number of animals.
[4] The t-tests are made between the mean of an experimental and that of its control group.
† The t-tests are made between active and hibernating groups under the same condition of captivity.

steroid release from the gland, as has been suggested for other species (6, 7, 8), then the resultant low adrenal vitamin value obtained for this stressed hibernator can be explained. A rapid release of adrenal steroid accompanied or preceded by ascorbic acid would leave behind in the tissue only minimal amounts, as the data in Table 1 indicate.

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ANNOUNCEMENTS

THE ENDOCRINE SOCIETY

The 44th Annual Meeting will be held June 21–23, 1962, Palmer House, Chicago, Illinois.

THIRTEENTH POSTGRADUATE ASSEMBLY IN ENDOCRINOLOGY AND METABOLISM

THE CLINICAL CENTER · BETHESDA MARYLAND

October 2–6, 1961

PROGRAM

CHAIRMEN: Dr. Roy Hertz and Dr. F. C. Bartter

Monday Morning, October 2

8:00– 9:00	Registration
	PITUITARY AND HYPOTHALAMUS. (Chairman: Dr. Charles W. Lloyd)
9:00– 9:35	Experimental Studies in Neuro-Endocrine Relationships—Dr. Evelyn Anderson
9:35–10:00	Chemistry of Pituitary Hormones—Dr. Peter Condliffe
10:10–10:45	Bioassay and Function of Pituitary Hormones—Dr. Robert Bates
10:45–11:00	Intermission
11:00–11:40	Clinical Disorders of Anterior Pituitary—Dr. Griff T. Ross
11:40–12:20	Clinical Disorders of Mid-Brain and Posterior Pituitary—Dr. Charles W. Lloyd
12:20– 1:00	Endocrine Effects of Hypophysectomy and Stalk Section in Man—Dr. M. B. Lipsett
1:00– 2:00	Lunch

Monday Afternoon

2:00– 5:00	Case Presentations. Panel and Discussion: Disorders of Mid-Brain, Posterior Pituitary and Anterior Pituitary—Drs. Lipsett, Lloyd, Bartter, Ross, McCullagh
5:30– 6:30	Cash Bar Cocktail Party and Reception—Navy Officers' Club

Tuesday Morning

	PARATHYROID DISORDERS. (Chairman: Dr. F. C. Bartter)
9:00– 9:30	Metabolic Effects of Parathyroid Hormone—Dr. F. C. Bartter
9:30–10:00	Hyperparathyroidism—Dr. F. R. Keating
10:00–10:30	Hypoparathyroidism—Dr. F. Engel
10:30–10:45	Intermission
10:45–11:15	Osteoporosis—Dr. Donald Whedon
11:15–11:45	Metabolic Bone Disease (other than Osteoporosis)—Dr. F. C. Bartter

11:45–12:15 Calcium Metabolism in Sarcoidosis—Dr. Norman Bell
12:15– 1:00 Panel and Case Presentations—Drs. Bartter, Keating, Engel, Whedon
1:00– 2:00 Lunch

Tuesday Afternoon

DISORDERS OF CARBOHYDRATE METABOLISM. (Chairman: Dr. E. Perry McCullagh)
2:00– 2:30 Carbohydrate Metabolism—Dr. DeWitt Stetten
2:30– 3:00 Insulin—Mechanisms and Assays—Dr. J. Field
3:00– 3:30 Clinical Uses and Mode of Action of Synthetic Hypoglycemic Agents—Dr. F. Lukens
3:30– 5:00 Panel—Cases—Drs. McCullagh, Lukens, Field, Engel

Wednesday Morning

PROBLEMS IN REPRODUCTION AND GONADAL FUNCTION. (Chairman: Dr. Roy Hertz)
9:00– 9:30 The Interpretation of Testicular Biopsies and Sex Chromatin Pattern Tests—Dr. W. O. Nelson
9:30–10:00 Metabolism Effects of Androgenic and Progestational Steroids—Dr. Richard Landau
10:00–10:30 Clinical Syndromes of Testicular Malformation and Dysfunction—Dr. E. P. McCullagh
10:30–10:45 Intermission
10:45–11:30 Sexual Precocity—Dr. Roy Hertz
11:30–12:15 Hirsutism, Stein-Leventhal and Related Syndromes—Dr. M. B. Lipsett
12:15– 1:00 Hormonal Relations between Mother and Fetus in Pregnancy—Dr. Claude Migeon
1:00– 2:00 Lunch

Wednesday Afternoon

PROBLEMS IN REPRODUCTION AND GONADAL FUNCTION (continued). (Chairman: Dr. Henry Turner)
2:00– 2:30 Gonadal Dysgenesis in Phenotipic Females—Dr. Henry Turner
2:30– 3:00 Secondary Amenorrhoea—Dr. Charles W. Lloyd
3:00– 3:30 Fertility and Sterility—Dr. Celso-Ramon Garcia
3:30– 5:00 Panel—Cases—Drs. McCullagh, Turner, Garcia, Migeon, Hertz, Lloyd
6:00 Cocktails (Cash Bar) Dinner for Registrants and Faculty, Navy Officers' Club

Thursday Morning

THYROID DISORDERS. (Chairman: Dr. Rulon Rawson)
9:00– 9:30 Experimental Thyroid Tumors—Dr. S. Wollman
9:30–10:30 Thyroid Cancer—Dr. Rulon Rawson
10:30–10:45 Intermission
10:45–11:15 Thyroxin Analogues—Their Properties and Uses—Dr. S. Asper
11:15–12:15 Evaluation of Medical Therapy of Thyroid Disease—Dr. F. R. Keating

12:15– 1:00 Thyroxin-Binding Proteins in Iodine Metabolism—Dr. J. Robbins
1:00– 2:00 Lunch

Thursday Afternoon

THYROID DISORDERS (continued). (Chairman: Dr. B. Dobyns)
2:00– 3:00 Indications and Procedures for Thyroid Surgery—Dr. B. Dobyns
3:00– 3:30 Autoimmunity Studies in Thyroid Disease—Dr. J. Robbins
3:30– 5:00 Cases and Panel—Drs. Rawson, Dobyns, Asper, Keating, Robbins

Friday Morning

ADRENAL DISORDERS. (Chairman: Dr. F. C. Bartter)
9:00– 9:30 Adrenal Physiology—Dr. C. Migeon
9:30–10:00 Tests of Adrenal Function—Dr. B. Kliman
10:00–10:30 Adrenal Insufficiency—Dr. J. Beck
10:30–10:45 Intermission
10:45–11:15 Hyperadrenocorticism with Respect to Hydrocortisone—Dr. F. Engel
11:15–11:45 Hyperadrenocorticism with Respect to Aldosterone—Dr. F. C. Bartter
11:45–12:15 Hyperadrenocorticism with Respect to Androgens—Dr. C. Migeon
12:15– 1:00 Panel and Cases—Drs. Bell, Engel, Migeon, Kliman, Beck, Bartter
1:00– 2:00 Lunch

Friday Afternoon

2:00– 3:00 Endocrine Aspects of the Cancer Problem—Dr. Roy Hertz
3:00– 3:45 Lipid Metabolism—Dr. D. Fredrickson

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GENERAL INFORMATION

Registration

8:00–9:00 A.M.—Auditorium, Clinical Center Building 10, National Institutes of Health.

Registration will be completed in advance by mail. Registrants will simply hand in their receipted registration card as they enter the Auditorium. Late registrants will be accommodated between 8:00 and 9:00 A.M., Monday, October 2.

Fee:

Course: $100.00 (Fellows and Residents $30.00).
Daily: $25.00.

This fee covers tutition, coffee breaks, Wednesday dinner for registrants and faculty

Housing

Block reservations are behind held until September 15 for course registrants and faculty at the following nearby motels:

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7740 Wisconsin Avenue
Bethesda, Maryland
Tel: OL. 6-2100

Intown Motor Hotel
Wisconsin Avenue and Bradley Boulevard
Bethesda, Maryland
Tel: OL. 4-1400

Individual registrants and faculty members must complete their own arrangements for housing by direct correspondence with these or other nearby motels. Cab service to NIH is available at nominal cost.

Housing arrangements are the individual's own responsibility. Reservations should be completed at least 1 month in advance.

Registration for the course should be completed by letter and check to:

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